# PROTEOMIC APPROACH TO INVESTIGATING THE MOLECULAR INTEGRITY OF INFLIXIMAB AFTER RECONSTITUTION AND DILUTION IN TYPICAL HOSPITAL CONDITIONS. (TCH034)


*Antonio Salmeron García[1], Natalia Navas Iglesias[2], José Cabeza Barrera[1], Antonio Lario Simón[3], M. Victoria Longobardo Polanco[3], Raquel Ortega López[2], Luís Fermín Capitán Vallvey[2]*

1 University Hospital San Cecilio, Hospital Pharmacy Unit, Granada, E-18012. Spain
2 University of Granada, Department of Analytical Chemistry, Faculty of Science, Campus Fuentenueva s/n, E-18071 Granada, Spain
3 CSIC (Consejo Superior Investigaciones Científicas), Proteomic Unit, Parasitology and Biomedicine Institute "Lopez Neira", Parque Tecnológico Ciencias de la Salud, Avda. del Conocimiento, s/n.18100 Armilla, Granada, Spain


## Objective

**Long term stability study of the molecular integrity of infliximab using peptide map fingerprinting when reconstituted and diluted in the typical hospital conditions**

## ANALYTICAL METHOD

**Trypsin digestion of Infliximab solutions :**

1.- Infliximab solutions were diluted with ammonium bicarbonate 50 mM to end up with 3 µg of protein.

2.- Reduction/alkylation of the disulfide bonds prior to trypsin addition:
- DTT (Dithiotheitol) 10 mM solution, incubation at 56 ºC for 45 minutes (reduction)
- Iodoacetamide solution 43 mM, incubation at room temperature for 30 minutes in the dark (alkylation).

*DigestPro, Intavis AG*

3.- Trypsin digestion:
- Trypsine Gold (MassSpectrometry Grade) 10ng/µL pH 8, incubation at 37 ºC for 4 hours.
- TFA (Trifluoroacetic acid) 0.2 % to stop digestion.

### MALDI-TOF-MS Analysis

1 µL of the infliximab digested was mixed with1 µL of the α-cyano matrix solution (α-CHCA 5 mg/ml 50 % acetonitrile and 0.1 % TFA) and 0.5µl were loaded on the MALDI target.


*MALDI-TOF mass spectrometer. Voyager DE-PRO from Applied Biosystem*

## STABILITY STUDY DESIGN

Infliximab (IFX) is a monoclonal antibody against tumour necrosis factor alpha (TNF-α). It is used for the treatment of psoriasis, Crohn's disease, ankylosing spondylitis, psoriatic arthritis, rheumatoid arthritis and ulcerative colitis.

According to the manufacturer's instructions, IFX must be prepared by reconstituting the contents of the vial (100 mg) by adding 10 mL of water for injectable preparations, so obtaining a solution of 10 mg/mL. This solution must be diluted with sodium chloride 0.9% up to 250 mL, obtaining varying concentrations depending on the dose required by the patient. The manufacturer recommends that the dilution should be used within 3 hours of preparation or within 24 hours if prepared in aseptic form and stored between 2 - 8 ° C. On the basis of these instructions, we studied the chemical integrity of IFX at different concentrations and storage conditions. 10mg/mL concentration samples were stored in glass vials and 0.5 and 2mg/ml in polyolefin bags (Viaflo ®), each sample was analyzed in quintuplicate.

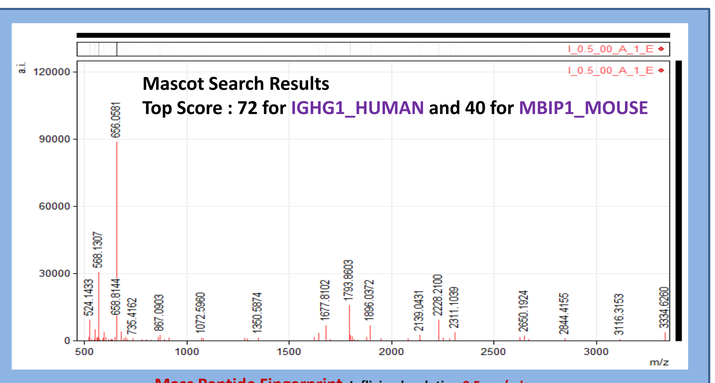
The storage conditions and the check times are described in the table below .

**Table 1. IFX solutions and storage conditions**

| Control Days | 0 | 1 | 3 | 4 | 7 | 14 | 21 | 28 | 44 | 58 | 73 | 88 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Storage | | | | | | | | | | | | |
| Room Temp. | 10/0.5/2 mg/ml | 10/0.5/2 mg/ml | | | | | | | | | | |
| 2 – 8 ° C | | 10/0.5/2 mg/ml | 10/0.5/2 mg/ml | 10/0.5/2 mg/ml | 10/0.5/2 mg/ml | 10 mg/ml | 10 mg/ml | 10 mg/ml | 10 mg/ml | 10 mg/ml | 10 mg/ml | 10 mg/ml |
| -20°C | | 10/0.5/2 mg/ml | 10/0.5/2 mg/ml | 10/0.5/2 mg/ml | 10/0.5/2 mg/ml | 10 mg/ml | 10 mg/ml | 10 mg/ml | 10 mg/ml | 10 mg/ml | 10 mg/ml | 10 mg/ml |

## RESULTS

### 0.5 mg/mL infliximab solution

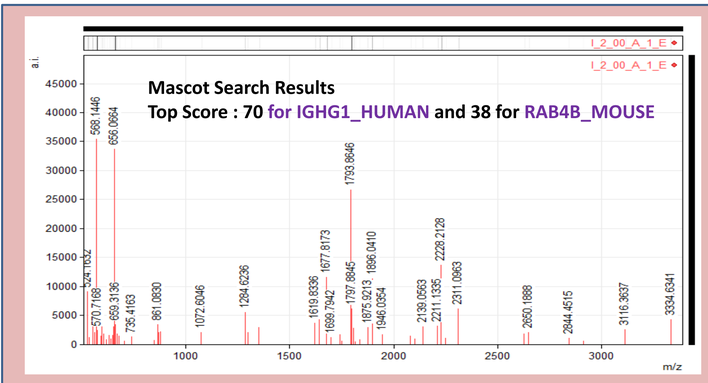


**Mass Peptide Fingerprint: Infliximab solution 0.5 mg/ml**
**The most representative peptides (73)**
**obtained immediately after preparation of the solution**

| Peptide | Amino acid sequence |
|---|---|
| 3334.6 (H) | SCDKTHTCPPCPAPELLGGPSVFLFPPKPK |
| 2844.4 (H) | THTCPPCPAPELLGGPSVFLFPPKPK |
| 2139.0 (H) | TPEVTCVVVDVSHEDPEVK |
| 1677.8 (H) | FNWYVDGVEVHNAK |
| 1808.0 (H) | VVSVLTVLHQDWLNGK |
| 2228.2 (H) | VVSVLTVLHQDWLNGKEYK |
| 735.4 (H) | CKVSNK |
| 656.4 (H) | AKGQPR |
| 735.4(M) | LQSLMK |
| 1677.8 (M) | AIHPTEDLQDEGKPK |
| 603.3(M) | AEIDR |
| 1793.8 (M) | TDAVFTPYPGFKSHVK |
| 1284.6 (M) | SREADSMAAHLP |

H: from IGHG1_Human / M: From RAB4B Mouse

### 2.0 mg/mL infliximab solution



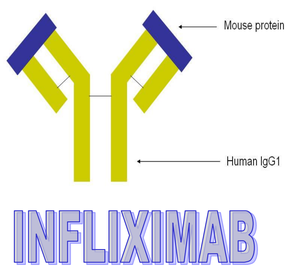
**Mass Peptide Fingerprint: Infliximab solution 2.0 mg/ml**
**The most representative peptides (67)**
**obtained immediately after preparation of the solution**

| Peptide | Amino acid sequence |
|---|---|
| 3334.6 (H) | SCDKTHTCPPCPAPELLGGPSVFLFPPKPK |
| 2844.4 (H) | THTCPPCPAPELLGGPSVFLFPPKPK |
| 2139.0 (H) | TPEVTCVVVDVSHEDPEVK |
| 1677.8 (H) | FNWYVDGVEVHNAK |
| 1808.0 (H) | VVSVLTVLHQDWLNGK |
| 2228.2 (H) | VVSVLTVLHQDWLNGKEYK |
| 735.4 (H) | CKVSNK |
| 2311.0 (M) | MAETYDFLFKFLVIGSAGTGK |
| 1916.8 (M) | LQIWDTAGQERFR |
| 1793.1 (M) | MGSGIQYGDISLRQLR |

H: from IGHG1_Human / M: From RAB4B Mouse

| Room temperature (24 h) | At 4 ºC (Fridge, 7 days) | At -20 ºC (Freezer, 7 days) |
|---|---|---|
| **29 %** of the most representative peptides were lost, including 603 (M), 656 (H) and 735 (H/M). Nevertheless, **protein identification** in the taxonomy was the same for **the IGHG1_HUMAN** (average score 70), but different for rodentia. | **33 %** of the most representative peptides were lost, including 603 (M) and 735 (H/M). Nevertheless, **protein identification** in the taxonomy was the same and with similar scores (69 for **IGHG1_HUMAN** and 40 for MBIP1_MOUSE) | **22 %** of the most representative peptides were lost, (including in this case only the peptide 603 (M)). Nevertheless, **protein identification** in the taxonomy was the same for **the IGHG1_HUMAN** (average score 70) but different for rodentia. |

Results at the end of the test period



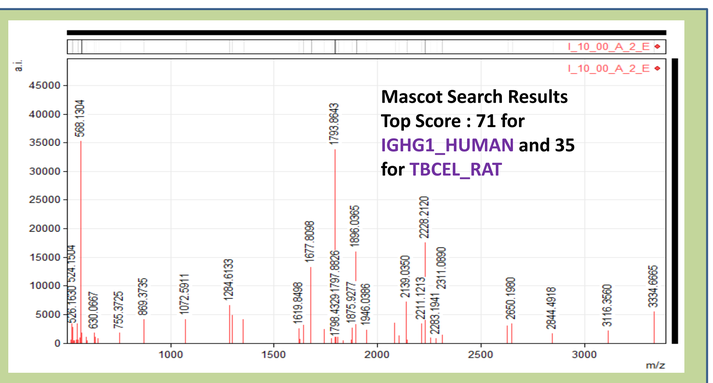

| Room temperature (24 h) | At 4 ºC (Fridge, 7 days) | At -20 ºC (Freezer, 7 days) |
|---|---|---|
| **19 %** of the most representative peptides were lost. Identified peptides from IGHG1_Human and from RAB4B Mouse were not lost. Nevertheless, **protein identification** in the taxonomy was the same for **the IGHG1_HUMAN** (average score 80) but different for rodentia. | **17 %** of the most representative peptides were lost. Identified peptides from IGHG1_Human and from RAB4B Mouse were not lost. Nevertheless, **protein identification** in the taxonomy was the same for the **IGHG1_HUMAN** (average score 70) but different for rodentia. | **13 %** of the most representative peptides were lost. Identified peptides from IGHG1_Human and from RAB4B Mouse were not lost. Nevertheless, **protein identification** in the taxonomy was the same for **the IGHG1_HUMAN** (average score 75) but different for rodentia. |

Results at the end of the test period

### 10.0 mg/mL infliximab solution

| Room temperature (24 h) | At 4 ºC (Fridge, 88 days) | At - 20 ºC (Freezer, 88 days) |
|---|---|---|
| **16 %** of the most representative peptides were lost. Identified peptides from IGHG1_Human and from TBCEL_RATRAB4B were not lost. **The protein identification score** was higher for the **IGHG1_HUMAN** (average score 90). Identification was different when rodentia taxonomy was tested. | **25 %** of the most representative peptides were lost, including peptide 1808 (H). Nevertheless, **protein identification** was higher for the **IGHG1_HUMAN** (average score 90), but was different when rodentia taxonomy was tested. | Only **4 %** of the most representative peptides (nºs 617 and 632) were lost. **Protein identification** was similar for the **IGHG1_HUMAN** (average score 70), but was different when rodentia taxonomy was tested. |

Results at the end of the test period



**Mass Peptide Fingerprint: Infliximab solution 10.0 mg/ml**
**The most representative peptides (56)**
**obtained immediately after preparation**

| Peptide | Amino acid sequence |
|---|---|
| 3334.6 (H) | SCDKTHTCPPCPAPELLGGPSVFLFPPKPK |
| 2844.4 (H) | THTCPPCPAPELLGGPSVFLFPPKPK |
| 2139.0 (H) | TPEVTCVVVDVSHEDPEVK |
| 1677.8 (H) | FNWYVDGVEVHNAK |
| 1808.0 (H) | VVSVLTVLHQDWLNGK |
| 2228.2 (H) | VVSVLTVLHQDWLNGKEYK |
| 1872(H) | EPQVYTLPPSRDELTK |
| 1797.8 (M) | DQPSGRSFMQVLCEK |
| 2311.0 (M) | SFMQVLCEKYSPENFPYR |
| 3334.6 (M) | KLGVMFPSLDTLVLANNHVNAIEEPADSLAR |

H: from IGHG1_Human / M: From RAB4B Mouse

## DISCUSSION

The mass peptide fingerprint of IFX obtained immediately after the preparation of the three different solutions we studied (Table 1) showed similar patterns, but each solution had a different number of representative peptides with a variability of up to 26 %. High degrees of variability are usual in these kinds of MALDI-TOF mass spectra due to the different processes that take place beforehand (enzymatic digestion, yield of the crystallization and ionization, etc). Nevertheless, **the same protein was always identified** with a high score value, i.e. **IGHG1_Human** (protein scores greater than 70 are significant). This result was expected since IFX is a chimeric IgG1 monoclonal antibody. The mourine part was not significant however in any of the cases, which means that no discussion could be based on it.

All the mass peptide fingerprints obtained during the test period for the three kinds of solution under the different storage conditions always lead to the **identification of the same protein, the IGHG1_Human** (scores greater than 70). In consecuence, and despite the variability (up to 33 %), it could be concluded that there were **no dramatic changes in the main part of the IgG1 structure and no chemical changes related with the cleavage sites**. We also confirmed that the peptides identified were the same (those shown with their sequences in the tables above)

## CONCLUSION

IFX solution of **10.0 mg/ml** (in water for injectable preparation), **2.0 mg/ml** (in sodium chloride 0.9 %) and **0.5 mg/ml** (in sodium chloride 0.9 %) refrigerated at 4 ºC or frozen at -20ºC **did not undergo dramatic chemical changes over the test period** (three months for the solution of 10.0 mg/ml and a week for the more diluted solutions). The samples stored for 24 hours at room temperature showed similar behaviour.

This study is part of a wider project that seeks to contribute to the establishment of practical stability studies of biotherapeutics based on monoclonal antibodies. Following several IHC guidelines, including ICHQ5C (stability testing of biotechnological/biological products), the chemical integrity of the IFX is currently being studied by size exclusion chromatography (SEC), and its biological stability by performing specific ELISA tests based on the TNF-α. By gathering all these results together, we can provide additional stability data covering practical uses of IFX. This also conforms to the recent recommendations of the "European conference consensus" sponsored by the French Society of Oncology Pharmacy (SFPO/ Annales Pharmaceutiques Françaises (2011) 69, 221—231).


## Acknowledgment

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