**Characterization of surfactant (polysorbate) inferences in biotherapeutics stability**.

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**Highlights**

* Separation of surfactant in drug using ODS Shim Pack MAYI ODS Column
* Characterize the separate surfactant using analytical orthogonal tools (HPLC, HPLC MS and GC MS)
* Observed the major changes in polysorbate structure and identified saturated and unsaturated fatty acids and the estimated levels may cause the stability in the biotherapeutics (mAb) shelf life.
* Identified critical attribute helps to optimize the level of surfactant (polysorbate) in biotherapeutics (mAb) stability.

**Introduction**

Polysorbates are the most widely used non-ionic surfactants to stabilize protein pharmaceuticals against interface- induced aggregation and surface adsorption. They are effective against various stresses such as agitation (for example, shaking or stirring), freeze/thawing and lyophilization. Nevertheless, some of their characteristics need to be carefully considered and monitored. Commercially avail- able polysorbates (PS) 20 and 80 are chemically diverse mixtures containing mainly sorbitan POE fatty acid esters. Additionally, substantial amounts of POE, sorbitan POE and isosorbide POE fatty acid esters are present. This leads to a significant degree of lot-to-lot variability requiring a close scrutiny of each lot in order to ensure uniform behavior. The presence of residual levels of peroxide in bulk polysorbate is also a concern. There have been reports of a buildup of peroxides in bulk as well as in aqueous solutions of polysorbate, when exposed to ambient oxygen and light. Depending on handling and storage conditions and supplier lot, varying concentrations of peroxides (9 ppm– 250 ppm) were noted among different lots of polysorbates . The buildup of peroxides can be detrimental not only to the stability of polysorbate itself but also to the protein therapeutic, which it stabilizes. Based on this consideration, Harmon et al. have also developed an oxidation stress test relying on peroxides formed in PS80 in the presence of Fe (III). polysorbate or by spiking degradation products resulting from oxidation such as fatty acids and fatty acid esters into protein formulations were investigated.

**Methods**

1. Surfactant separation using Shim-pack MAYI Column.

Shim-pack MAYI (5 mm L. × 2.0 mm I.D., 50 μm)column in this macromolecular proteins are blocked and cannot enter the pores, smaller molecules infiltrate the chemically modified pores to be retained on the column. By incorporating this column with another column by switching HPLC flow lines, proteins introduced into the pre-treatment (C18 Reverse phase, 100 mm L. × 2.1 mm I.D., 5 μm) column from the autosampler are directly discharged out of the system after passing through the column and using a UV detector (wavelength 280 nm) to monitor an mAb molecule. For details

Table No.1

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| --- | --- | --- |
| **column**  | Shim-pack MAYI-ODS (5 mm L. × 2.0 mm I.D., 50 μm)  | Zorbax 5u C18 100 Å, (100 mm L. × 2.1 mm I.D., 5 μm) |
| **Mobile phase**  |  A: 10 mmol/L Ammonium Formate in Water B: 2-Propanol  | C: 10 mmol/L Ammonium Formate in Water D: 2-Propanol |
| **Time program** |  Solvent switching, A (0 - 1.5 min) → B (1.5 - 3.5 min) → A (3.5 - 9 min)  | D.Conc. 5 % (0 - 1 min) → 100 % (6 - 7 min) → 5 % (7.01 - 9 min) |
| **Flowrate** |  0.6 mL/min  | 0.3 mL/min |
| **Extraction time** | 1 min |  - |
| **Injection Vol** | 10 ul |  - |
| **Column Temp** | 40 ̊C  | 40 ̊C  |
| **Detection**  | UV 280 | 3200 QTRAP (LC MS), ESI Positive Mode, 50 to 1750 M/Z Scan Mode |

Due to the weak UV absorption of polysorbate, a mass spectrometer was used for detection in the analytical flow line. Generally, polysorbate includes a large number of by-products, and because some of these are very strongly retained, 2-propanol was used as the final mobile phase.

1. Analysis of extracted surfactant (polysorbate) using Gas chromatography mass spectrometry (GC MS)

Gas Chromatography Mass spectrometry (GC MS) analysis of extracted (polysorbate) samples were performed using Agilent 7890A series GC system equipped with a Supelco Omega wax column (30m\*0.25mm ID, 0.25um thickness, Sigma) coupled with a Agilent 7000 QQQ MS. Omega wax column was used for good separation and for gas chromatography mass spectroscopic detection. Electron ionization system with ionization energy of 70eV was used, 99.99 % pure helium gas was used as a carrier gas at a constant flow rate of 1.1 ml/min. mass transfer line and injector temperature were set at 220˚C and 250˚C respectively, and the oven temperature was programmed, initial temperature was 150 °C for 1 min then 10 °C/min to 240 °C for 15 min with total 25 min run. 1 ul of sample injected in the split mode 10:1. the signals were recorded in full scan mode (m/z 20-600, 250 scan/milli seconds). All components were identified by comparison of theirs mass spectra with those obtained from authentic samples and/or the NIST mass spectral database using AMDIS and mass hunter software.

**Results and discussion**

Separation of mAb using shim Pack MAYI ODS and C18 Zorbax column.



**Fig.1** mAb peak separate from surfactant



**Fig 2**. Surfactant peak separated from mAb using Pre-treatment column

A

B

C

D

E

**Fig. 3** LC MS data, A. Total ion chromatograph (TIC), B. mAb 1 mass spectrum, C. mAb 2 mass spectrum, D. mAb 3 mass spectrum , E. mAb 4 mass spectrum. The above mass spectrum shows the separation of free fatty acids in polysorbate complex and it is showing doubly and triply charge of free fatty acids.











**Fig. 4** GC MS data, Gas chromatograph for polysorbate 80 (standard), mAb 1, mAb2, mAb3 and mAb 4 respectively, The above chromatograph shows the separation of free fatty acids which is showing both saturated and unsaturated fatty acid profile.

**Table No.2**

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Other than stable fatty acids from PS 80 cause reduce the surfactant concentration for stability and it cause the effect on shelf life of the protein and destabilization of protein. saturated fatty acids are less effect to compare to the unsaturated fatty acids levels on stability.

Effect of polyunsaturated fatty acids (PUFA) (C16:1; C18:1; (C18:2n-7,9 trans,cis) ; (C18:2) ; (C18:2n-7,10) ; (C22:1n-9) occurs on mAbs lifetimes and this binding effect on decreasing immunoreactivity with monoclonal antibodies spanning amino acid residues. and this may cause the precipitation and visible particles in PS 80 degradation and this effects instability to the mAb. Interaction propensity of the carboxylate moiety of the fatty acid with the mAb coupled with the low solubility of the fatty acid leading to destabilizing effects. the level of degradant fatty acid reduces the concentration of surfactant and may lead to destabilizing the mAb.

**Conclusions**

The formulations for biopharmaceuticals invariably comprise of Polysorbates to prevent aggregation and surface adsorption. However, polysorbates consist of trace levels of peroxides as impurities that could catalyze target product degradation via oxidation. In this work, we used HPLC-UV and mass spectrometry to catalogue the polysorbate 80 and its derivatives in commercial biosimilar products. Spiked samples with varying levels of the polysorbate 80 impurities were studied for their impact on the structure and function of the target product. The study reveals that polysorbates in the marketed products vary considerably and the implicate the quality of the product, in several instances. Future work proceeding to Simulations and molecular docking studies provide insights for engineering the product so that the ensuing structural and functional changes are minimized.

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