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Short communication

A simple reversed phase high-performance liquid chromatography method for polysorbate 80 quantitation in monoclonal antibody drug products

Michael Adamo[c], Lawrence W. Dick Jr.[a], Difei Qiu[a], An-Horng Lee[a], John Devincentis[b], Kuang-Chuan Cheng[a,*]

[a] Analytical Development, Medarex, Inc., A Wholly Owned Subsidiary of Bristol-Myers Squibb, 519 Route 173 West, Bloomsbury, NJ 08804, United States
[b] Bristol-Myers Squibb, 6000 Thompson Rd., Syracuse, NY 13057, United States
[c] Bristol-Myers Squibb, 311 Pennington-Rocky Hill Road, Pennington, NJ 08534, United States

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ABSTRACT

In this paper, we discuss an improved high-performance liquid chromatography (HPLC) method for the quantitation of polysorbate 80 (polyoxyethylenesorbitan monooleate), a commonly used stabilizing excipient in therapeutic drug solutions. This method is performed by quantitation of oleic acid, a hydrolysis product of polysorbate 80. Using base hydrolysis, polysorbate 80 releases the oleic acid at a 1:1 molar ratio. The oleic acid can then be separated from other polysorbate 80 hydrolysis products and matrices using reversed phase HPLC. The oleic acid is monitored without derivatization using the absorbance at 195 nm. The method was validated and also shown to be accurate for the quantitation of polysorbate 80 in a high protein concentration monoclonal antibody drug product. For the measured polysorbate 80 concentrations, the repeatability was less than 6.2% relative standard deviation of the mean (% RSD) with the day-to-day intermediate precision being less than 8.2% RSD. The accuracy of the oleic acid quantitation averaged 94–109% in different IgG_1 and IgG_4 drug solutions with variable polysorbate 80 concentrations. In this study, polyoxyethylene, a by-product of the polysorbate 80 hydrolysis was also identified. This peak was not identified by previous methods and also increased proportionally to the polysorbate 80 concentration. We have developed and qualified a method which uses common equipment found in most laboratories and is usable over a range of monoclonal antibody subclasses and protein concentrations.

1. Introduction

Polysorbate 80 and other polysorbates are commonly used as stabilizing excipients in biopharmaceutical formulations [1]. In protein formulations, polysorbates minimize adsorption to surfaces, reduce the rate of protein denaturation and increase the drug solubility and stability [1,2]. Due to this critical role, accurate quantitation of polysorbate 80 is needed to assure product quality.

Polysorbate 80 is an oleate ester of sorbitol and its anhydrides copolymerized with 20 moles of ethylene oxide for each mole of sorbitol and sorbitol anhydride (Fig. 1). Due to its molecular heterogeneity and lack of a good chromophore, polysorbate 80 in its native form cannot be accurately analyzed by the conventional method of HPLC with UV absorbance detection [3]. Direct quantitation of polysorbate 80 by various other methods has been published in literature. Analysis of polysorbate 80 by high-performance liquid chromatography (HPLC) with evaporative light scattering detection (ELSD) was reported [3] but there was no mention of the drug protein concentrations used and the quantitation limit of the method was higher than the value reported here. Other methods also included (HPLC) with electrospray mass spectrometry (ESI) [4,5]. These methods analyzed polysorbate 80 in plasma samples and may not be suitable for analysis of polysorbate 80 in high protein concentration samples. Size exclusion chromatography with UV absorbance detection of polysorbate 80 has also been reported in the literature but showed a decrease in the polysorbate 80 quantitation limit when analyzed in the presence of protein [6]. Polysorbate 80 has also been analyzed by colorimetry but it required a multi-step sample preparation including solid-phase extraction and did not obtain the limit of quantitation reported in this work [7]. Although these methods allow for the direct quantitation of polysorbate 80 they could not obtain an equivalent quantitation limit as reported in this work or required extensive sample preparation, skilled analysts and/or specialized equipment.

Polysorbate 80 can be hydrolyzed at the ester linkage under basic conditions to release oleic acid at a 1:1 molar ratio. The oleic

* Corresponding author. Tel.: +1 908 479 2422; fax: +1 908 479 2402.
E-mail address: kcheng@medarex.com (K.-C. Cheng).

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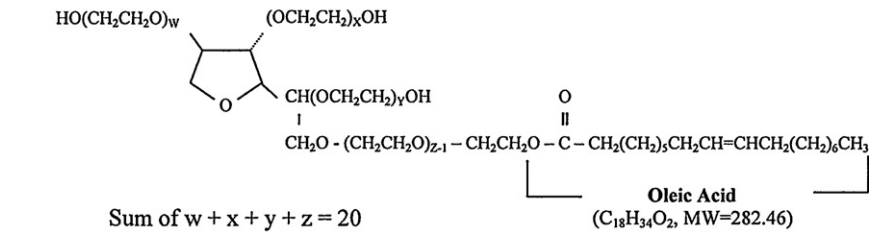
Polysorbate 80



Nonadecenoic Acid

$[CH_3(CH_2)_7CH{=}CH(CH_2)_8COOH]$ ($C_{19}H_{36}O_2$, MW = 296.49)

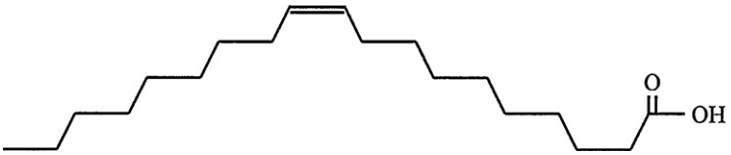


Fig. 1. Structures of polysorbate 80, nonadecenoic acid and oleic acid.

acid can then be analyzed by standard RP-HPLC methods with detection by UV absorbance. Using the free fatty acid from hydrolysis of polysorbates for their quantitation has been previously reported [8–10]. The methods which allowed for the quantitation of polysorbate 80 were either investigated primarily in the pharmaceutical suspension without protein [9] or with protein concentrations less than 35 mg/mL [10]. This paper describes an improved RP-HPLC method using alkali hydrolysis and standard UV detection for the quantitation of polysorbate 80 in the presence of high protein concentration samples (up to 75 mg/mL). This method incorporates an extraction step which can increase the usable life of the column since sodium hydroxide and any unhydrolyzed proteins from the high protein concentration samples will be not be injected on the system. This method is also able to separate oleic acid from polyoxyethylene. None of the other methods identified this polyoxyethylene peak which also increased proportionally to the polysorbate 80 concentration. The peak identities of oleic acid and polyoxyethylene were confirmed by mass spectrometry analysis. This method was validated and used successfully for the quantitation of polysorbate 80 down to 20 ppm in a range of monoclonal antibody subclasses and at protein concentrations up to 75 mg/mL.

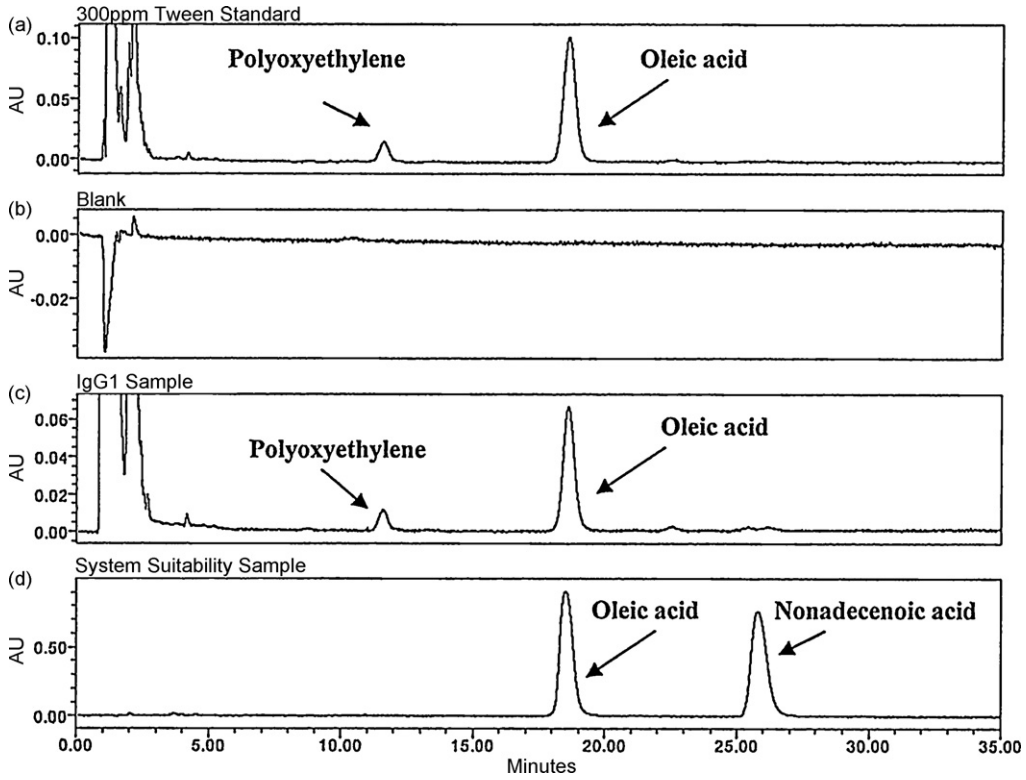


Fig. 2. A hydrolyzed polysorbate 80 sample compared to oleic acid, nonadecenoic acid and a formulation blank chromatogram. (a) Polysorbate 80 standard; (b) formulation buffer blank; (c) IgG_1 formulated with polysorbate 80; and (d) system suitability sample containing oleic and nonadecenoic acids.

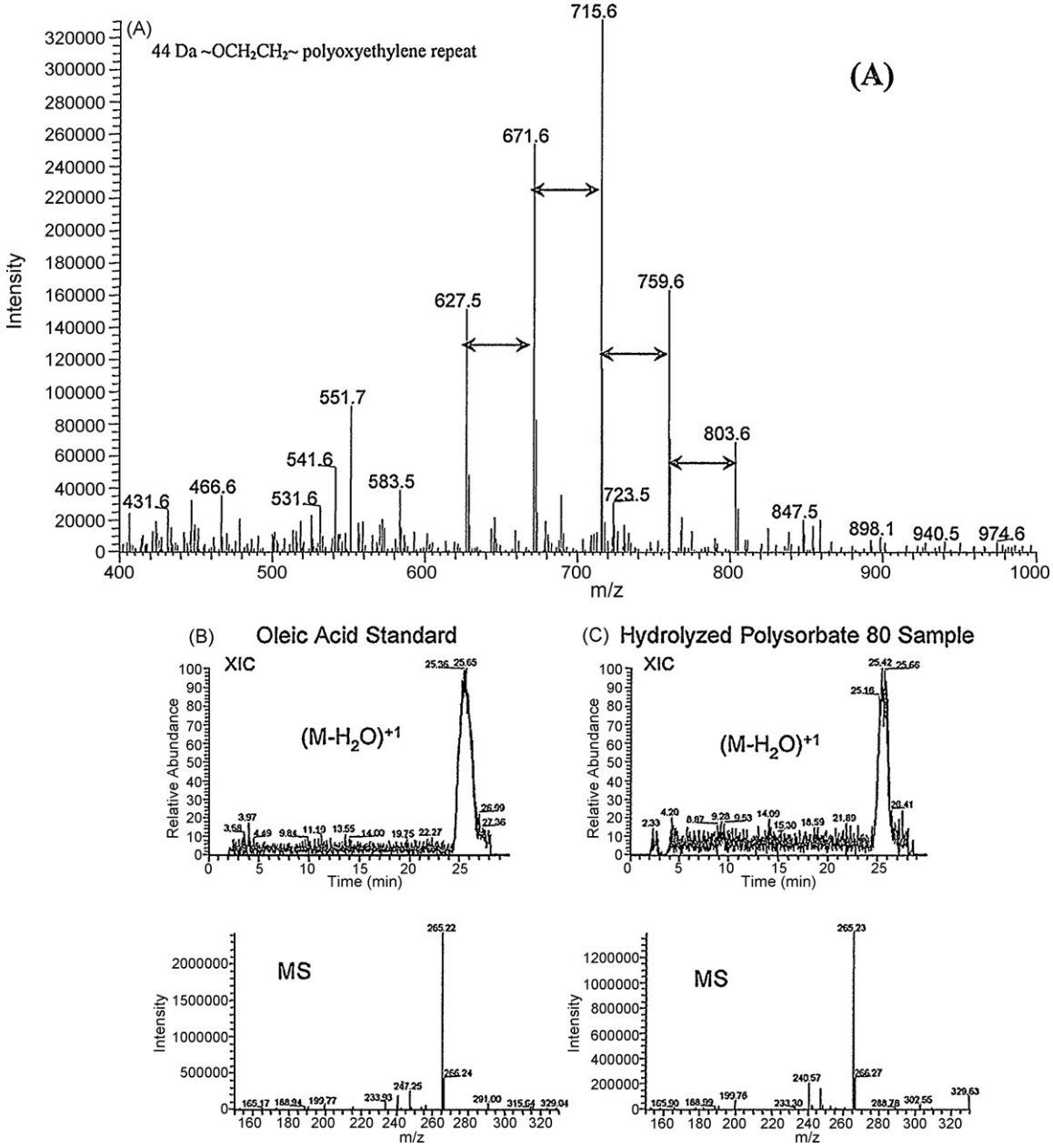


Fig. 3. Mass spectral confirmation of (A), the polyoxyethylene peak in the hydrolyzed polysorbate 80 sample (B), the oleic acid peak in the oleic acid standard and (C), the oleic acid peak in the hydrolyzed polysorbate 80 sample. Samples were analyzed on an Agilent 1100 HPLC coupled to a Thermo Finnigan LCQ Deca XP with an APCI interface.

2. Experimental

2.1. Apparatus

Determination of the polysorbate 80 content in the formulated product was performed using a Waters 2695 separation module with a 2996 photodiode array detector (Waters Corporation, Milford, MA). The injection volume was 100 μL. The sample temperature was set to 25 °C and the column temperature was 30 °C. The flow rate was set to 1 mL/min using an absorbance of 195 nm for detection. The detection was performed at 195 nm since the greatest response for the oleic acid was obtained at that wavelength and no interference from any other components in the hydrolyzed samples was observed. The oleic acid was separated on a Waters Symmetry C18, 5 μm, 3.9 mm × 150 mm column with a Waters Symmetry C18, 5 μm, 3.9 mm × 20 mm guard cartridge by isocratic elution for 25 min with 80:20, acetonitrile:20 mM potassium phosphate monobasic (adjusted to a pH of 2.8 with H_3PO_4 on an Accumet AR20 pH meter).

Confirmation of the oleic acid and polyoxyethylene peaks by mass spectrometry was performed using an Agilent 1100 series HPLC coupled to a Thermo Finnigan LCQ Deca XP with an APCI interface. The oleic acid was separated on a Waters Symmetry C18, 5 μm, 3.9 mm × 150 mm column by isocratic elution using 90% Solvent B (0.09% TFA in ACN) with 10% Solvent A (0.1% TFA in water). The column temperature was set to 25 °C. The flow rate was set to 200 μL/min. The source heater temperature was 450 °C and the capillary temperature was 200 °C. The MS detector was set to scan a mass range of *m*/*z* 150–330 for the oleic acid peak and *m*/*z* 400–1000 for the polyoxyethylene peak.

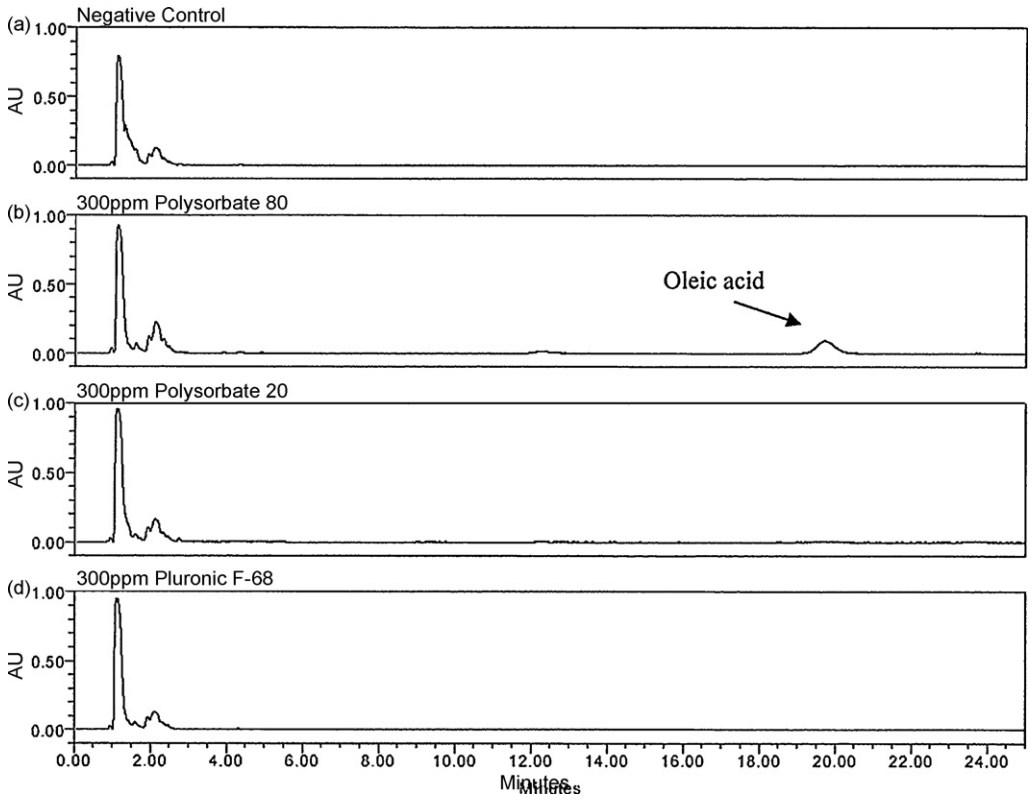


Fig. 4. Method specificity as demonstrated by comparison of the chromatographic profiles from the following samples: (a) Formulation buffer sample without polysorbate 80; (b) polysorbate 80 standard; (c) polysorbate 20 standard; and (d) Pluronic F-68 standard.

2.2. Reagents and solvents

The polysorbate 80, N.F. and 5 M NaOH were purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). Polysorbate 20, acetonitrile, 1 M NaOH and 85% phosphoric acid were from Fisher Scientific (Waltham, MA). The potassium phosphate monobasic was from VWR (West Chester, PA). High purity deionized water purified by a Millipore Milli-Q system (Billerica, MA) with a resistivity of 18.2 MΩ cm was used for buffer and sample preparation. Nonadecenoic acid was purchased from Matreya, LLC (Pleasant Gap, PA). Oleic acid and Pluronic F-68 were from Sigma–Aldrich (Saint Louis, MO). The 0.09% TFA in acetonitrile and 0.1% TFA in HPLC grade water were purchased from Burdick & Jackson (Morristown, NJ).

2.3. Method parameters

Due to a variable content of oleic acid in different lots of polysorbate 80, the same lot of polysorbate 80 used in the spiking of the monoclonal antibody (MAb) drug samples was also used to construct the calibration curve for quantitation. For the preparation of the standards a 100,000 ppm (w/w) stock polysorbate 80 was prepared. This 100,000 ppm stock solution was diluted to a 1000 ppm (v/v) working solution. The working solution was further diluted (v/v) to concentrations of 750, 500, 300, 200, 150, 100, 50 and 20 ppm for construction of the calibration curve for quantitation. The standards were processed similarly to the samples. The system suitability sample was a 1:1 (v/v) mixture of oleic acid and the structurally similar nonadecenoic acid. A system suitability test was performed concomitantly with the test sample analysis to show that the RP-HPLC method is repeatable by comparison of the relative retention time of oleic acid to nonadecenoic acid in replicate injections.

The IgG_4 monoclonal antibody sample was prepared by spiking 3 μL of a 10,000 ppm polysorbate 80 stock solution into 297 μL of the sample. The IgG_1 monoclonal antibody sample was prepared by spiking 6 μL of a 10,000 ppm polysorbate 80 stock solution into 294 μL of the sample. The non-fucosylated IgG_1 monoclonal antibody sample was prepared by spiking 12 μL of a 10,000 ppm polysorbate 80 stock solution into 288 μL of the sample. 300 μL of the polysorbate 80 spiked MAb samples and polysorbate 80 standards were hydrolyzed by adding 300 μL of 300 mM sodium hydroxide and then incubating at 60 °C for 18 h to release oleic acid. Upon cooling of the reaction vial to room temperature, 150 μL of 5 M sodium hydroxide and 600 μL of acetonitrile were added. The addition of 5 M sodium hydroxide post-hydrolysis was used to force phase separation of the aqueous-acetonitrile mixture. The mixture is biphasic with the upper acetonitrile layer containing the analyte, oleic acid. 200 μL of the acetonitrile layer was collected for analysis, to which 50 μL of 20 mM potassium phosphate was added. The polysorbate 80 concentration in the sample was determined by RP-HPLC, via the analysis of oleic acid peak at 195 nm.

2.4. Assay performance

System suitability was established by analysis of the relative retention time between oleic acid and the structurally similar nonadecenoic acid by multiple injections over six runs. The structure of polysorbate 80, oleic acid and nonadecenoic acid are illustrated in Fig. 1.

The assay specificity for polysorbate 80 in the presence of Pluronic F-68 and polysorbate 20 as potential matrix components was assessed.

The polysorbate 80 calibration curve was expanded to range from 5 to 1000 ppm for determination of the linearity. The linearity was determined by analysis of the coefficient of determination (R^2) from triplicate injections of each standard.

The detection limit (DL) and quantitation limit (QL) were calculated based on the slope of a specific calibration curve using

Table 1
Repeatability, intermediate precision and accuracy of oleic acid RP–HPLC retention time and polysorbate 80 concentration measurements.

	Day 1			Day 2			Day-to-day		
	R_t (min)[a]	Calc. conc. (ppm)[a]	% Recovery[a]	R_t (min)[a]	Calc. conc. (ppm)[a]	% Recovery[a]	R_t (min)[b]	Calc. conc. (ppm)[b]	% Recovery[b]
IgG_4 monoclonal antibody	19.52 ± 0.008	106 ± 1.97	106 ± 1.97	19.33 ± 0.010	98 ± 4.13	98 ± 4.13	19.43 ± 0.099	102 ± 5.12	102 ± 5.12
IgG_1 monoclonal antibody	19.53 ± 0.007	215 ± 4.09	107 ± 2.04	19.33 ± 0.014	187 ± 11.41	94 ± 5.71	19.43 ± 0.100	201 ± 16.37	100 ± 8.19
Non-fucosylated IgG_1 monoclonal antibody	19.51 ± 0.010	436 ± 6.75	109 ± 1.69	19.31 ± 0.013	382 ± 18.74	96 ± 4.68	19.41 ± 0.105	409 ± 31.08	102 ± 7.77

[a] Average value from six injections ± SD.
[b] Average value from twelve injections ± SD.

multiple concentrations of hydrolytically released oleic acid in the range of the QL, and its residual standard error of the regression line. The DL and QL are calculated using the following expressions: DL = (3.3σ)/*Slope* and QL = (10σ)/*Slope*. Where slope is obtained by linear regression of the specific dose–response calibration curve and σ is the standard error of the regression line.

Accuracy of the method was evaluated by determining the recovery of fortified polysorbate 80 in six replicate analyses (six separate preparations each with a single injection) of an IgG_4, IgG_1 and non-fucosylated IgG_1 monoclonal antibody by a single analyst over two days.

Precision (repeatability and intermediate precision) was ascertained by analysis of the oleic acid retention time and determined concentration of polysorbate 80 fortified in an IgG_4, IgG_1 and non-fucosylated IgG_1 monoclonal antibody. Six separate preparations of each sample by a single analyst were analyzed on two separate days.

The protein concentration limit of the assay was evaluated by analysis of an unformulated IgG_1 monoclonal antibody sample at protein concentrations of 25, 50 and 75 mg/mL fortified with 500 ppm polysorbate 80.

3. Results and discussion

The average relative retention time of oleic acid (t_R = 19.24 ± 0.16 min) to nonadecenoic acid (t_R = 26.85 ± 0.25 min) in the system suitability samples from six separate assays ranged from 0.715 to 0.717 with <0.1% relative standard deviation of the mean (RSD). Fig. 2 contains a typical sample and standard chromatogram compared to the oleic acid, nonadecenoic acid and formulation blank traces. The hydrolyzed polysorbate 80 standard and sample both had a peak around 12 min. Further characterization of this peak by mass spectrometry analysis shows a structure of polyoxyethylene (–O–CH_2–CH_2–) with a repeat 44 dalton difference between peaks (Fig. 3A). This polyoxyethylene peak also increases proportionally to the polysorbate 80 concentration. The results shown here demonstrate the methods ability to differentiate oleic acid from polyoxyethylene (a hydrolysis product of polysorbate 80) and nonadecenoic acid which has a similar structure and MW to oleic acid.

Confirmation of the oleic acid peak identity was achieved by mass spectrometry. The extracted ion chromatogram (XIC) from an injection of an oleic acid standard was compared to that of the alkali hydrolysis sample of polysorbate 80 (Fig. 3B and C). Both the standard and sample contained a peak at 25 min with an m/z of 265 daltons which corresponds to oleic acid with a loss of a water molecule.

The alkali hydrolysate of formulation buffer, 300 ppm Pluronic F-68 and 300 ppm polysorbate 20 showed a distinctly different peak pattern from that of alkali digest of the 300 ppm polysorbate 80 standard (Fig. 4). No oleic acid was observed in the closely related surfactant polysorbate 20 or the Pluronic F-68. This simple method clearly shows specificity to differentiate polysorbate 80 from other surfactants such as polysorbate 20 or from Pluronic F-68 which is commonly used in upstream cell culture for production.

The polysorbate 80 dose–response was shown to be linear in the concentration range of 5 to 1000 ppm ($y = 11141x + 33620$) with a coefficient of determination (R^2) of 0.9995. The % RSD for triplicate injections at each concentration point ranged from 1.0 to 13.7%. For QL and DL determination, a specific calibration curve in the concentration range of 5 ppm to 80 ppm ($y = 11500x + 6157.5$) with an R^2 value of 0.9974 was used in the regression analysis. The slope of the polysorbate 80 dose–response curve was determined as 11,500, and the standard error of the regression line was shown as 14,625. Thus, the limits were calculated as 4 ppm for DL and 13 ppm for QL.

The repeatability of the method was calculated as % RSD. The repeatability for day 1 was 0.04–0.05% RSD for retention time and 1.55–1.90% for polysorbate 80 concentration in ppm (Table 1). The repeatability for day 2 was 0.05–0.07% RSD for retention time and 4.21–6.10% RSD for polysorbate 80 concentration (Table 1).

The intermediate precision expressed as % RSD for the oleic acid peak retention time and polysorbate 80 concentration (ppm) ranged from 0.51% to 0.54% and 5.02% to 8.14%, respectively (Table 1). The polysorbate 80 concentration in the samples ranged from 100 to 400 ppm in both the repeatability and intermediate precision studies.

Accuracy of the method was evaluated by determining the recovery of fortified polysorbate 80 in six different preparations of monoclonal IgG_1 and IgG_4 drug formulations at three concentration levels on two separate days. The accuracy of the method ranged from 94% to 109% (Table 1). The average recovery of polysorbate 80 in the three MAb samples was 102 ppm in the 100 ppm sample, 201 ppm in the 200 ppm sample and 409 ppm in the 400 ppm sample (Table 1).

Recovery of polysorbate 80 from an IgG_4 MAb sample at protein concentrations of 25, 50 and 75 mg/mL was evaluated. Triplicate injections of each sample were analyzed and the average percent recovery of polysorbate 80 in the samples ranged from $107 \pm 0.6\%$ at 25 mg/mL, $103 \pm 2.0\%$ at 50 mg/mL and $98 \pm 2.9\%$ at 75 mg/mL. MAb protein concentrations above 25 mg/mL initially formed a gel when the NaOH was added. This gel-like reaction mixture dissolved at the hydrolysis temperature used and had no effect on the recovery of polysorbate 80 in the samples.

4. Conclusion

This improved RP-HPLC method has been shown to be precise and accurate for the quantitation of polysorbate 80 in formulated MAb drug solutions up to 75 mg/mL. It requires minimal sample preparation, standard UV detection and is sensitive, with the method limits being 4 ppm for DL and 13 ppm for QL. The detection and quantitation limits are well below those needed to measure the polysorbate 80 content in most drug substance or drug products. This method is able to separate oleic acid from other hydrolysis products (*i.e.* polyoxyethylene) that could affect quantitation if not resolved. This method requires common equipment found in the laboratory and is easily transferable between laboratories and sites. Previous polysorbate 80 hydrolysis methods [9,10] which did not employ a phase separation were not adequate for sample analysis. After a few injections of the high protein concentration samples the column performance was deteriorated (data not shown). This may have been due to the sodium hydroxide in the sample or protein degredants created during the sample processing. By forcing a phase separation we have devised a method which is more robust than those previously mentioned. With the biopharmaceutical industry trending towards higher concentration formulations, this method provides a quick and accurate analysis of polysorbate 80 in protein concentrations up to 75 mg/mL.

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