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Fast and sensitive determination of Polysorbate 80 in solutions containing proteins

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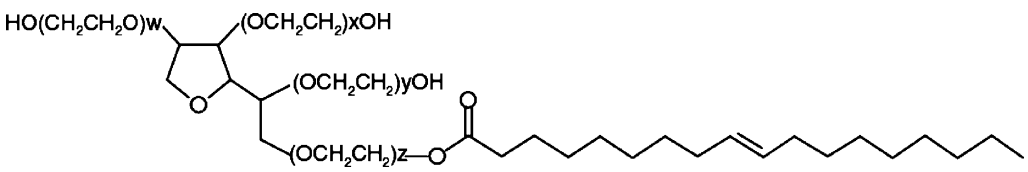
ABSTRACT

A fast and sensitive method has been developed for the specific determination of Polysorbate 80 (Tween 80) in liquid formulations in the presence of proteins and excipients. The quantitative determination is based on a fast liquid chromatographic (HPLC) separation and charged aerosol detection (CAD). The method was validated using a Poroshell 300SB-C18 column packed with 5 μm shell particles (75 mm × 2.1 mm) and acetonitrile–methanol–water–trifluoroacetic acid mobile phase at a flow rate of 0.65 ml/min. The rapid LC–CAD method is suitable for quantifying Polysorbate 80 in the range of 10–60 μg/ml in protein solutions within good manufacturing practices (GMPs) of the pharmaceutical industry.

1. Introduction

A common feature of many protein formulations is the presence of added surfactants (such as Tween 20 and Tween 80). The reason for their application is to retain the biological activity of proteins by maintaining a specific three-dimensional conformation upon storage, transportation and delivery to patients. In liquid pharmaceutical formulations, surfactants are added to minimize protein adsorption to surfaces (containers and syringes) and to reduce the air–liquid, solid–liquid interfacial surface tension in order to decrease the rate of protein denaturation that can lead to aggregation [1–3]. Proteins at the interface can unfold further, exposing more hydrophobic surface in order to enhance amphiphilicity. Surfactants fall into several categories based on their molecular charge: cationic, anionic, zwitterionic and non-ionic. Non-ionic surfactants include polyoxyethylene sorbitans, polyoxyethylene ethers and polyethylene–polypropylene glycols. To quantify some commonly used non-ionic surfactants in various protein formulations may be extremely difficult because of their heterogeneous molecular composition and non-chromophoric characteristics.

The polysorbates are amphiphilic, non-ionic surfactants composed of fatty acid esters of polyoxyethylene sorbitan. Polysorbate 20 (PS-20, polyoxyethylenesorbitan monolaurate) and Polysorbate 80 (PS-80, polyoxyethylenesorbitan monooleate) are the most common polysorbates currently used in the formulation of protein biopharmaceuticals. Both types of polysorbates have a common backbone and only differ in the structures of the fatty acid side-chains. The hydrocarbon chains provide the hydrophobic nature of the polysorbates while the hydrophilic nature is provided by the ethylene oxide subunits. In solution the polysorbates occur as either monomers or in micelles depending on a number of factors including the polysorbate concentration, buffer composition, and temperature of the solution [4,5]. Polysorbate 80 (Tween 80) has a chemical formula of:



where $W+X+Y+Z \approx 20$.

The number of possible molecular structures is greater than 1500, the average molecular mass is approximately 1300 g/mol.

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Regulatory agencies are increasingly asking for methods to quantify the amount of surfactants and other ingredients in the final product. Commonly used quantitative methods for Polysorbate 80 are quite time consuming (derivatization) and use hazardous solvents [6–9]. Tani et al. applied a method using size exclusion chromatography with mobile phase containing the surfactant to be determined (Polysorbate 80) above its critical micellar concentration (CMC). The presence of micellar surfactant in the mobile phase shortens the elution time of the surfactant resolving it from low-molecular mass excipients and, in many cases, proteins (Mr < 50 000). The method requires the use of mixed micellar solutions in order to alter the CMC of the surfactant of interest or resolve the micellar peak from interfering protein or excipients. Polysorbate 80 standards showed linearity from 2 to 1000 pg/ml [10]. A kinetic spectrophotometric method for the determination of Tween 80 based on its interaction with 5(*p*-dimethylaminobenzylidene)rhodanine (PDR) in alkaline media was reported by Pourezza and Rastegarzadeh [11].

Charged aerosol detection (CAD) is a relatively new technique. This is why it is not so widespread yet in pharmaceutical analysis; only few applications can be found in the literature [12]. CAD is based upon using an electrical aerosol analyzer coupled to HPLC system [13,14]. It operates by detecting charged particles that have a selected range of mobility rather than by measuring individual gas-phase ions that are differentiated based upon mass-to-charge ratio (*m*/*z*) [15]. The mobile phase is nebulized using nitrogen as the carrier gas, followed by the droplets in the aerosol shrinking as the solvent evaporates. This stream of particles from the nebulizer is met with a second stream of positively charged nitrogen gas from a high-voltage platinum corona charger in a mixing chamber, where the positive charge from the nitrogen gas is transferred to the particles. The amount of charge acquired is directly proportional to particle size. The charge is then transferred to a collector where it is measured by a highly sensitive electrometer, generating a signal in direct proportion to the quantity of analyte present.

The speed of chromatographic separation can be increased with different approaches. One opportunity to get higher efficiency is to reduce the diffusion path in a packed column.

The concept of superficial or shell stationary phases was introduced by Horvath et al. [16,17]. They applied 50 μm glass bead particles covered with styrene-divinylbenzene based ion exchange resin, became known as pellicular packing material. Later Kirkland described, that 30–40 μm diameter superficially porous packings (1 μm phase thickness, 100 Å pores) provided much faster separations, compared with the large porous particles used earlier in liquid chromatography [18]. Afterward the core diameter was reduced and the thickness of active layer was cut to 0.5 μm and was used for fast separation of peptides and proteins [19]. The most recent introduction of a superficially porous particle is the so-called fused-core particle [20,21]. Wide-pore, superficially porous, silica-based packings with solid 4.5-μm cores and a 0.25-μm porous outer layer (Poroshell) demonstrate a variety of characteristics that are beneficial for the separation of proteins and macromolecules [22].

The aim of this study was to demonstrate the applicability of column packed with partially porous wide-pore particles for fast separation of macromolecules (proteins) and CAD detection for immediate determination of Polysorbate 80 by developing, validating and applying an LC/CAD method. This general concept can be applied in routine analysis for many protein formulations. The selectivity of RP-LC based separation can be easily adjusted by varying the mobile phase composition (gradient program) and by changing the separation temperature. The present study shows an example of the direct fast determination of Polysorbate 80 from an injection solution containing a four-helix bundle protein which belongs to the family of cytokines.

2. Experimental

2.1. Solvents and reference material

Acetonitrile, methanol (Gradient grade), trifluoroacetic acid (Uvasol), hydrogen peroxide 30% (Pro analysi) and hydrochloric acid 1N (Titripur), were purchased from Merck (Darmstadt, Germany). Deionized water was freshly prepared using a Milli-Q® equipment (Milli-Q gradient A10 by Millipore). The reference material as Polysorbate 80 (preservative free, low peroxide and low carbonyls), L-methionine (Reagent grade) and 2,5-dihydroxybenzoic acid (Puriss, matrix substance for MALDI MS) were purchased from Sigma–Aldrich Ltd., Budapest. Dithiothreitol (PlusOne DTT) was obtained from BioRad Ltd., Budapest.

2.2. Equipment, column

Throughout the separations an Agilent 1200 system with Chemstation software from Kromat Ltd., Budapest, Hungary, was employed. The system was equipped with a photodiode array detector in line with a corona charged aerosol detector (ESA Inc., Chelmsford, MA). A Poroshell 300SB-C18 column with a particle size of 5 μm (75 mm × 2.1 mm) was used (Kromat Ltd., Budapest).

The molecular mass distribution of Polysorbate 80 was measured with a Shimadzu (Biotech) Axima Confidence MALDI–TOF mass spectrometer (MS) using a Shimadzu AccuSpot NSM1, Nano Scale Spotter for MALDI–TOF MS (Simkon Kft, Hungary, Budapest).

2.3. Optimization of the chromatographic conditions

For method development, the mobile phases were prepared by mixing at different ratios HPLC gradient grade acetonitrile, methanol, Milli-Q water and trifluoroacetic acid. The mixtures were degassed by sonication for 5 min. The composition of organic phase was varied between 100% acetonitrile and acetonitrile:methanol 50:50 (v/v) in 10% steps.

The stock solutions of reference standards (Polysorbate 80) were dissolved in water (1000 μg/ml), then it was diluted to 500 μg/ml for scouting gradient runs and 40 μg/ml as reference test solutions for polysorbate content determination. Protein solutions containing 40 μg/ml Polysorbate 80 were also applied for method development to find the suitable chromatographic conditions for the specific separation of peaks of polysorbate origin. The protein solution was injected directly without dilution. The protein in this example was a cytokine, which shares a common four-helix-bundle architecture, and lacks glycosylation. The protein contains native disulfide bonds. The protein content of the test solution was 1 mg/ml.

Linear gradients with 8, 16 and 32 min gradient span at different column temperatures (20, 40 and 60 °C) with different flow rates (0.40, 0.55 0.70 ml/min) were run for systematic method development. Gradient program, column temperature, mobile phase composition, injection volume, flow rate and detector (CAD) parameters (such as range and nebulizing temperature) were optimized applying a fractional factorial experimental design (Statistica 9 software was used to find the optimal condition).

Injecting 500 μg/ml Polysorbate 80 showed that reverse-phase separation and CAD detection can distinguish many components of polysorbates (Fig. 1). Two major peak groups of Polysorbate 80 can be detected and can be the target compounds of polysorbate determination from solutions containing low level of Polysorbate 80. The first peak group (peak pair) is eluted at 18–20 min while the more hydrophobic major components are eluted at 29–32 min retention range during the 32 min long scouting gradient. The more polar major peak pair (at 18–20 min retention time in Fig. 1) gave the most intensive signal of all peaks of polysorbate origin

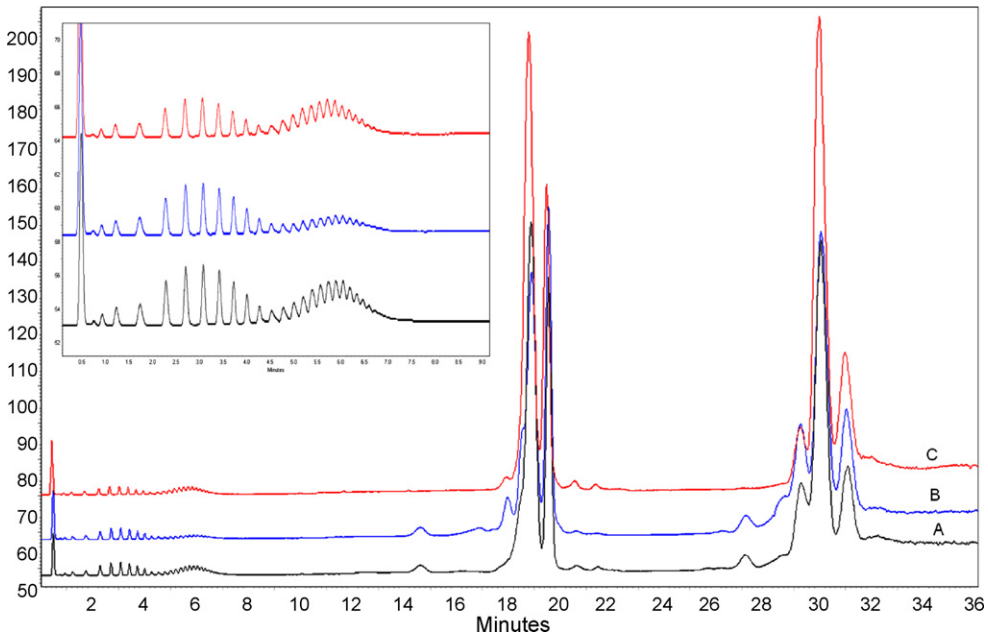

Fig. 1. Scouting – charged aerosol detected (CAD) – chromatograms of 500 μg/ml Polysorbate 80. A, B and C chromatograms illustrate three different batches of Polysorbate 80. Chromatographic conditions: Poroshell 300SB-C18 column packed with 5 μm shell particles (75 mm × 2.1 mm), mobile phase: acetonitrile–methanol–water–trifluoroacetic acid gradient elution (0–100% "B", in 32 min), flow: 0.70 ml/min, column temperature: 20 °C, injection volume: 5 μl, detection: charged aerosol detection, range: 50 pA, nebulizing temperature: 30 °C, gas (nitrogen) pressure: 37–39 psi.

when it was diluted to 40 μg/ml. In the protein formulation the expected concentration of Polysorbate 80 is 40 μg/ml. For further method development only the diluted polysorbate and the protein solutions were used. The results obtained during the method development showed that resolution and selectivity between the peaks of polysorbate and protein origin can be improved applying low column temperature such as 20–30 °C (Fig. 2). Other beneficial parameter is the mobile phase composition. Adding 10–30% methanol as organic modifier beside the acetonitrile enhances the efficiency (sharper peaks) and thus the resolution can be increased.

2.4. Optimized HPLC–CAD conditions for Polysorbate 80 determination

The experimental design was evaluated in terms of separation speed and selectivity. The shortest possible separation is about 8–10 min with sufficient selectivity and resolution. It is significantly shorter than it can be found in the literature. The size exclusion chromatographic method of Tani requires a 45 min separation [10].

The optimized method uses a Poroshell 300SB-C18 column packed with 5 μm shell particles (75 mm × 2.1 mm) and acetonitrile–methanol–water–triflouroacetic acid mobile phase

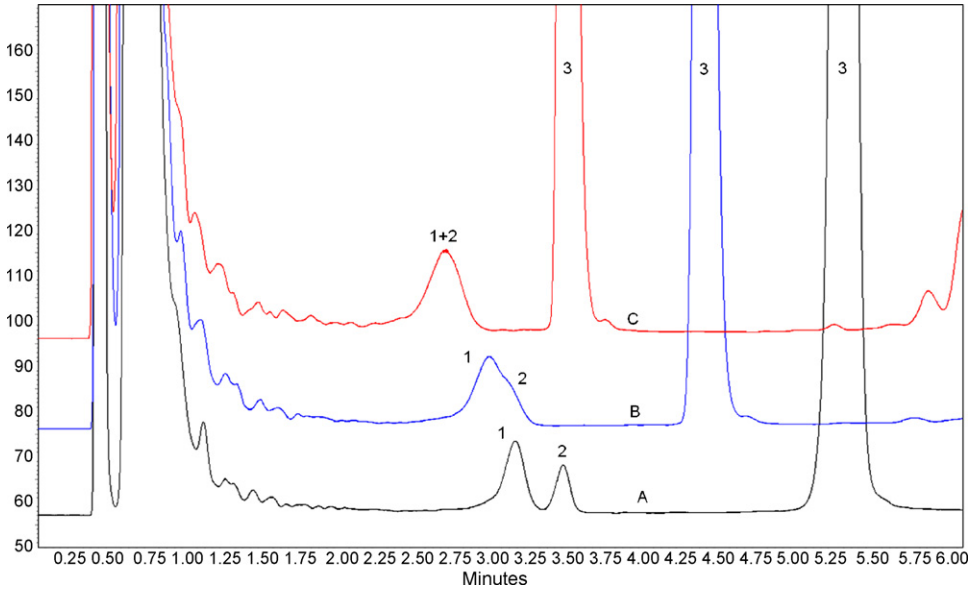

Fig. 2. Chromatograms of protein solution containing 1 mg/ml protein and 40 μg/ml Polysorbate 80. Chromatographic conditions: Poroshell 300SB-C18 column packed with 5 μm shell particles (75 mm × 2.1 mm), mobile phase: acetonitrile–methanol–water–trifluoroacetic acid gradient elution (50–100% "B", in 6 min), flow: 0.65 ml/min, column temperature: 20 °C (A), 40 °C (B) and 60 °C (C), injection volume: 5 μl, sample temperature: 4 °C detection: charged aerosol detection, range: 50 pA, nebulizing temperature: 30 °C, gas (nitrogen) pressure: 37–39 psi. Peaks: 1, 2: major peaks of Polysorbate 80 origin and 3: protein (cytokine).

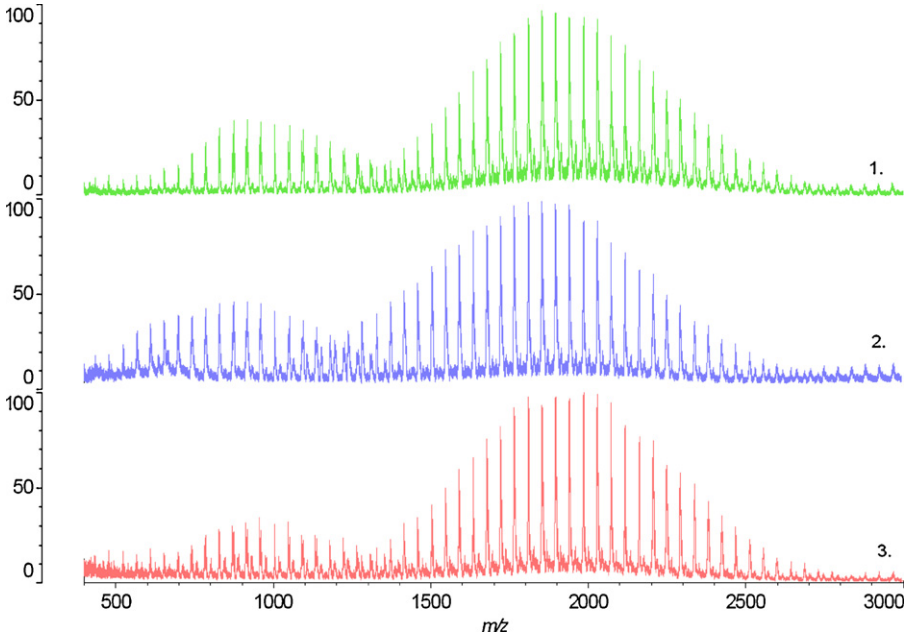

Fig. 3. MALDI–TOF MS spectra of three different Polysorbate 80 batches (Sigma–Aldrich, preservative free, low peroxide, low carbonyls). Matrix substance: 2,5-dihydroxybenzoic acid (10 mg/ml) in acetonitrile–water–trifluoroacetic acid (50:50:0.1), sample preparation: 0.5 μl matrix solution and 0.5 μl, 500 μg/ml Polysorbate 80 solution). The spectra were collected in linear positive mode.

gradient at a flow rate of 0.65 ml/min. Mobile phase "A" consists of acetonitrile–methanol–water–triflouroacetic acid with 80:20:900:1 (v/v) ratio, and mobile phase "B" with 720:180:100:1 (v/v) ratio. The gradient program starts with 50% "B" eluent, and a 6 min gradient duration is applied up to 100% "B" and it is kept till 7 min. After this gradient elution program the column is re-equilibrated (post-run) with the starting mobile phase composition for 3 min. The injection volume is set to 5 μl. The detector parameters are set as follows; range: 50 pA, nebulizing temperature: 30 °C, gas (nitrogen) pressure: 37–39 psi. The sample compartment is thermostated at 4 °C to avoid protein degradation or aggregation.

2.5. Mass spectrometric measurements

During the method development stage mass spectrometric (MS) detection was also used in addition to CAD. The aim of MS measurements was to determine which substances eluted in the polysorbate main peak. Preliminary measurements were performed with matrix-assisted laser desorption/ionisation-time of flight (MALDI–TOF) MS to determine the mass distribution of Polysorbate 80. The matrix substance (2,5-dihydroxybenzoic acid) was dissolved (10 mg/ml) in acetonitrile–water–triflouroacetic acid (50:50:0.1). Then the matrix solution was mixed with the 500 μg/ml Polysorbate 80 solution on the target plate (0.5 + 0.5 μl). The spectra were collected in linear positive mode in the range of 400–3000 Da. Fig. 3 shows the obtained MS spectra of three different polysorbate batches. Significant differences in mass distribution can be observed however all three batches show similar bimodal distribution.

The separation of all polysorbate isomers is not possible with reverse-phase chromatography thus MS detection was applied to conclude which polysorbate components are coeluted in the first, most polar main chromatographic peak, which was chosen as the target compound of polysorbate determination (t_r = 3.1 min in Fig. 2A). Applying a splitter built in after the column, the mobile phase flow rate was splitted (in 1:99 ratio) toward the CAD detector (99% flow) and to the AccuSpot (1% flow) to collect a fraction of the target polysorbate peak on a MALDI plate. Then the spectrum of the collected sample was obtained (Fig. 4). It can be seen that the major target peak contains at least 15 different polysorbate compounds in the mass range of 1300–2000 Da.

3. Results and discussion

3.1. Method validation

A fast, gradient reverse-phase HPLC method with charged aerosol detection has been developed to separate and determine peaks of Polysorbate 80 origin and matrix compounds, and can be applied for in process control (IPC) analysis for routine work. The selectivity (or even elution order) of peaks of Polysorbate 80 origin and peaks of protein origin can be changed significantly with varying the column temperature or changing the ratio of methanol and acetonitrile in the mobile phase. It is very useful when Polysorbate 80 is measured in different matrixes (different protein solutions).

The HPLC–CAD method was validated. The determination of Polysorbate 80 concentration can be based on the first, most polar main peak of Polysorbate 80. The method validation was performed in accordance with the recent guidelines [23–25].

3.1.1. Specificity

To prove that the determination of Polysorbate 80 is selective and free from any disturbing effects, reference solutions, protein containing solutions and stressed protein (oxidized, reduced and deamidated) solutions were examined.

The protein used in this study contains several methionines in its sequence. Hydrogen peroxide oxidation of two methionines is relatively fast in the case of this protein. Oxidation at both methionine results in alterations of protein structure that affects the apparent molecular size and polarity, thus can be separated from the native molecule with reverse-phase liquid chromatography. Therefore it was necessary to verify that oxidized protein forms do not disturb the polysorbate determination, and baseline separation can be achieved between the polysorbate main peak and oxidized protein peaks (Fig. 5). The oxidation was performed by adding 1% (v/v) hydrogen peroxide (30%) into the protein solution, and after incu-

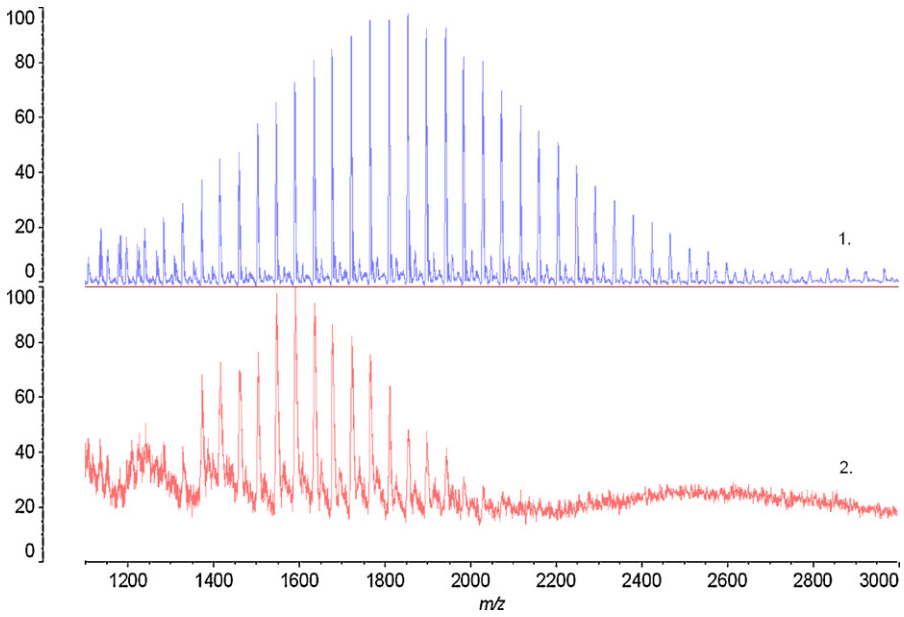

Fig. 4. MALDI–TOF MS spectra of Polysorbate 80 (1) and the MALDI–TOF MS spectra of main polysorbate peak after liquid chromatographic separation (2). Matrix substance: 2,5-dihydroxybenzoic acid (10 mg/ml) in acetonitrile–water–trifluoroacetic acid (50:50:0.1), sample preparation: 0.5 μl matrix solution and 0.5 μl, 500 μg/ml Polysorbate 80 solution). The spectra were collected in linear positive mode.

bating at 30 °C for 2 h the oxidation was stopped by adding 0.5 mg methionine to the solution.

Reduction of the protein can also affect the polysorbate determination. The model protein has intramolecular disulfide bonds. The disulfide bonds in this protein were reduced with dithiothreitol (DTT). 0.5 mg of DDT was added to 1 ml protein solution then it was incubated at 30 °C for 60 min. The protein reduction yielded a more hydrophobic compound than the native form (Fig. 5).

Deamidation of asparagine and glutamine in proteins results in a free carboxylic acid group. This process is accompanied by changes in polarity and hydrophobicity, mass and charge. Changes in polarity and hydrophobicity can be followed using RP-HPLC. In this example the RP-HPLC chromatogram showed a small amount of a less polar product after deamidation. In Fig. 5 chromatogram B shows a very small peak after the major protein peak. Deamidation was achieved by adding 100 μl hydrochloric acid (1N) to 900 μl protein solution. Then the mixture was incubated at 30 °C for 3 h.

Resolution of Rs » 1.5 was achieved between the polysorbate main peak (target peak, t_r = 3.1 min in Fig. 5) and the protein peaks or peaks of protein impurities or degradation products, therefore

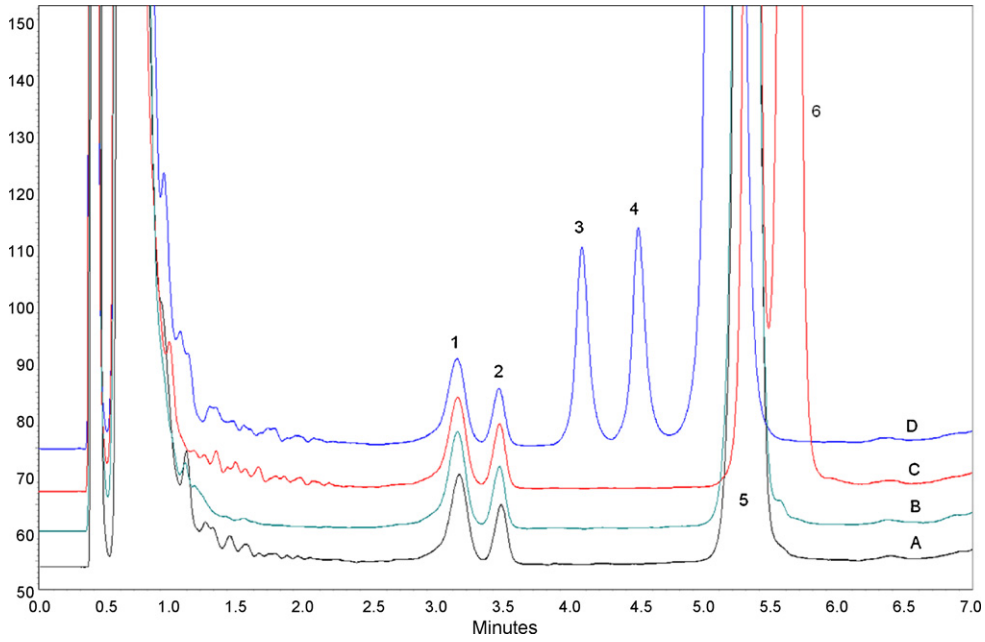

Fig. 5. Chromatograms of stressed protein solutions containing 1 mg/ml protein and 40 μg/ml Polysorbate 80. Chromatograms: protein solution (A), protein solution containing deamidated protein (B), protein solution containing reduced form (C), and protein solution containing two major oxidized protein form (D). Peaks: 1, 2: major peaks of Polysorbate 80 origin, 3, 4: oxidized protein, 5: protein (native) and 6: reduced protein form. Chromatographic conditions: Poroshell 300SB-C18 column packed with 5 μm shell particles (75 mm × 2.1 mm), mobile phase: acetonitrile–methanol–water–trifluoroacetic acid gradient elution (50–100% "B", in 6 min), flow: 0.65 ml/min, column temperature: 20 °C, injection volume: 5 μl, sample temperature: 4 °C detection: charged aerosol detection, range: 50 pA, nebulizing temperature: 30 °C, gas (nitrogen) pressure: 37–39 psi.

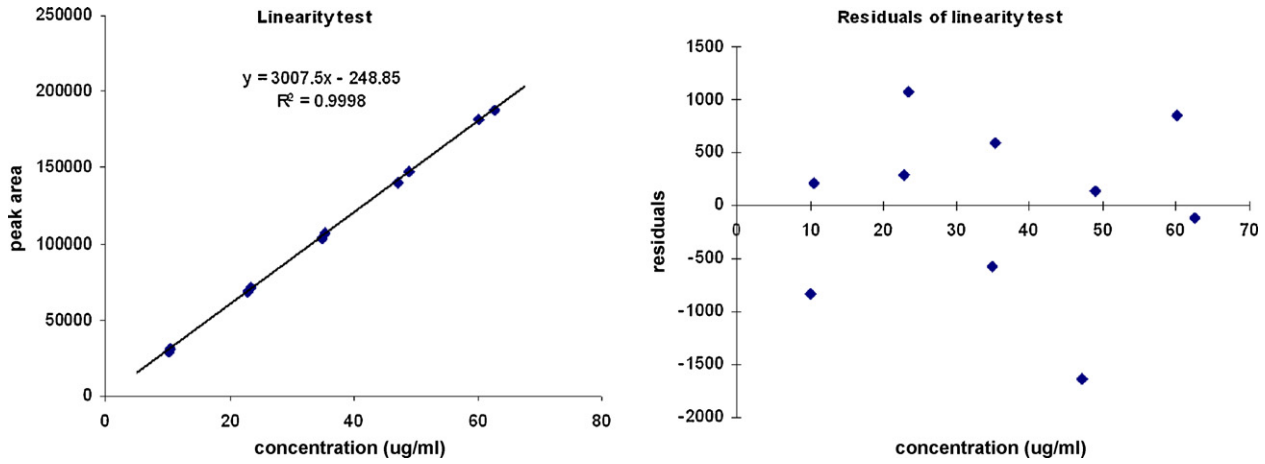

Fig. 6. Linearity test: fitted curve and residuals.

the method can be considered as a specific method for Polysorbate 80 determination. Fig. 5 shows the chromatograms obtained through the specificity study.

3.1.2. Linearity of response

The linearity of response was assessed by injecting reference standards prepared in sample solvent. The concentration range of compounds was investigated from the quantitation limit (LOQ) up to the 150% of the theoretical polysorbate content (range: 10–60 μg/ml Polysorbate 80). Ten independent results were obtained. The results were analyzed by linear regression. The correlation coefficients, r^2, were found $r^2 > 0.99$ ($r^2 = 0.9998$), confidence interval of "Y" intercept ($P = 95\%$) contained the origin (−1657 to 2972) and the residuals plotted uniformly and randomly around the regression line. The equation of fitted curve was: $y = 3008x - 249$. Fig. 6 shows the fitted curve and the residuals of linearity test.

3.1.3. Accuracy

Samples for recovery test were prepared as follows: Reference material (Polysorbate 80) in the range of LOQ—150% of theoretical value was spiked to protein solutions which did not contain Polysorbate 80. Polysorbate was pipetted from a stock solution (dissolved in water) then the solutions were homogenized (1 min shaking).

Sample concentrations were determined by reference to a calibration line constructed from standards containing the respective analyte in LOQ—150% around the theoretical Polysorbate 80 concentration. Ten independent results were obtained. The results were analyzed by linear regression. The correlation coefficients (r^2) were found $r^2 > 0.99$ ($r^2 = 0.9995$), confidence interval of "Y" intercept ($P = 95\%$) contained the origin (−0.96 to 0.47 μg/ml), the confidence interval ($P = 95\%$) of slope contained the value of 1 (0.99–1.02 μg/ml) and the residuals plotted uniformly and randomly around the regression line. The equation of fitted curve was: $y = 1.0055x - 0.243$. Fig. 7 shows the fitted curve and the residuals of accuracy test.

3.1.4. Precision, intermediate precision

Precision was examined by the relative standard deviation (RSD) of simultaneously prepared samples ($n = 6$). The RSD % of six analyses should be lower than 10% criteria was applied. For evaluating the intermediate precision the analysis was repeated ($n = 6$) on another day and the obtained results were compared by *t*-test and *F*-test. No significant differences were observed between the two repeated analyses. The RSD % obtained for precision study were RSD = 2.5% (first day) and 2.1% (second day). The obtained *F* value for intermediate precision test was found as $F = 1.68$ ($F_{critical} = 5.05$), while *t*-test resulted a value of $t = 2.09$ ($t_{critical} = 2.23$).

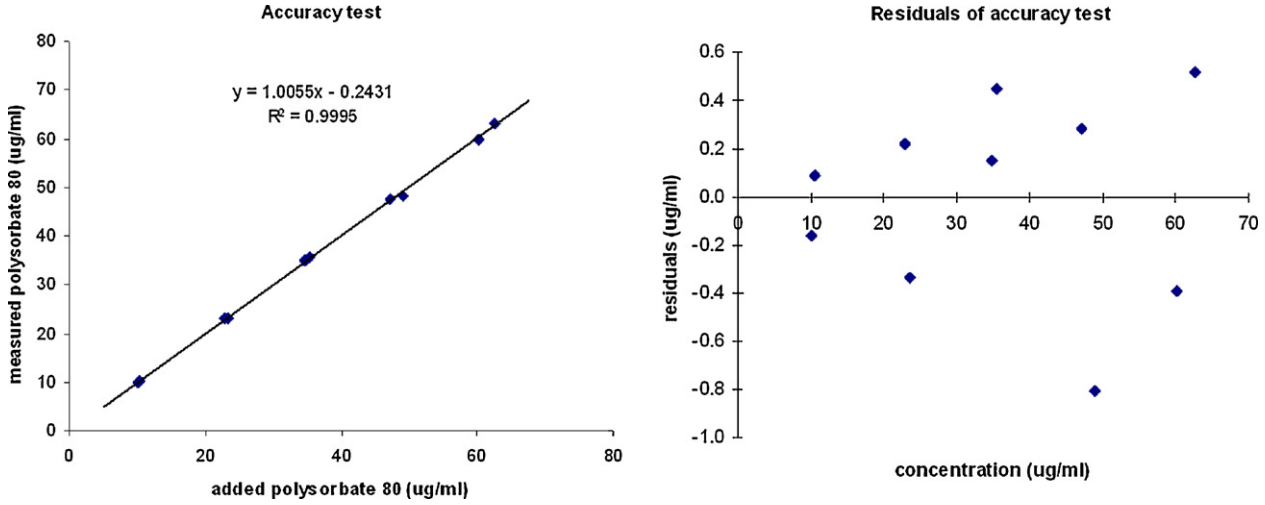

Fig. 7. Accuracy test: fitted curve and residuals.

3.1.5. Limit of quantitation and detection

Quantitation limit (LOQ) and detection limit (LOD) were determined by the RSD of peak areas obtained by five repeated injections (of standard solutions). The RSD <10% for LOQ concentration and RSD <30% for LOD concentration criteria were considered for this purpose. The obtained RSD of peak areas was 8.7% in the case of 10 μg/ml Polysorbate 80 solution while the 5 μg/ml Polysorbate 80 solution yielded a 23.8% RSD of five repeated injections. As a result 10 μg/ml was considered as LOQ and 5 μg/ml as LOD.

3.1.6. Stability of sample and stock solutions

The stability of sample solution was studied on standard as well as on test-sample (protein containing) solution at the concentration of nominal value (40 μg/ml Polysorbate 80). The solutions were stored in a sample compartment and were chromatographed 25 times within a 48-h period. RSD of peak areas was obtained, and linear regression was calculated for the fitted curve. The confidence interval (P = 95%) of slope contained the value of zero (−0.01 to 0.03) and the residuals plotted uniformly and randomly around the regression line. The obtained RSD during the 48-h test was smaller than 2% (1.9%). The standard and test solutions were proved to be stable for 48 h. There were no detectable degradants on the chromatograms during the stability study.

For the establishment of the stability of standard stock solution, three standard stock solutions were prepared and stored in refrigerator for 1 week. For the measurements freshly diluted solutions were used and injected every day, and the differences from the peak areas injected at the beginning were calculated. No more than 3% difference was observed each day (the highest deviation was 2.6%). The stock solutions of the reference substance were considered to be stable, for at least 7 days.

3.2. Determination of Polysorbate 80 from real samples

For routine measurement of Polysorbate 80, the determination is based on the peak area of the first main peak of Polysorbate origin. This peak elutes at t_r = 3.1 min (Fig. 5). The calculation is based on external standard method. The average peak area obtained from three parallel weighing of standard Polysorbate 80 solutions is used as reference peak area. The stock solutions of reference standards (Polysorbate 80) are dissolved in water (1000 μg/ml), and then they are diluted to 40 μg/ml as reference solutions. The test samples are injected without dilution. Two repeated injections of both samples and reference solutions are performed. The quantity of Polysorbate 80 can be calculated according to the next formula:

$$C_{\text{sample}} = \frac{A_{\text{sample}}}{A_{\text{ref}}} \frac{w}{V} D \times 1000$$

where C_{sample} is the Polysorbate 80 content of test solution (μg/ml), A_{sample} is the average peak area obtained by injecting the test solution, A_{ref} is the average peak area of reference solutions, w is the weighed amount (mg) of reference material (average of three weighing), V is the volume (ml) of reference stock solution, D is the dilution of reference solution and 1000 is the factor between mg and μg.

4. Conclusion

On the basis of this study, the use of fast chromatographic technique (applying partially porous packings) coupled with charged aerosol detection for the quantification of Polysorbate 80 from protein solutions is practical. The time reducing and solvent saving characteristics of the fast separation is very advantageous, compared to the most widely used conventional HPLC technique. Generally the separation of polysorbate main compounds from peaks of protein origin can be achieved within 10–15 min depending on the characteristics of protein and other excipients (matrix components). The selectivity of RP-LC based separation can be easily adjusted by varying the mobile phase composition (gradient program) and by changing the separation temperature.

Applying CAD detection does not necessitate the use of a mass spectrometry detector, which is more expensive. CAD detection is also used for polyethyleneglycol and Polysorbate-20 determination routinely in our practice. This technique can be constructive for the determination of other non-chromophoric excipients. The concept presented in this study can be applied in routine analysis for many protein formulations.

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