ELSEVIER

Journal of Chromatography A, 786 (1997) 99–106

JOURNAL OF CHROMATOGRAPHY A

Single step method for the accurate concentration determination of polysorbate 80

Travis H. Tani[a], Jamie M. Moore[b], Thomas W. Patapoff[b,*]

[a]*University of Michigan Medical School, Department of Biological Chemistry, Box 0606, Ann Arbor, MI 48109, USA*
[b]*Pharmaceutical Research and Development, Genentech, Inc., So. San Francisco, CA 94080, USA*

Received 11 March 1997; received in revised form 7 May 1997; accepted 7 May 1997

Abstract

A simple high-performance liquid chromatography (HPLC) method is described that has the potential of determining the concentration of numerous classes of surfactants in the presence of protein and excipients. The method consists of a size-exclusion column and mobile phase containing a surfactant at concentrations above the critical micelle concentration for that surfactant. 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 (M_r<50 000). Polysorbate 80 standards show linearity from 2 to 1000 μg/ml and have a coefficient of variation of less than 10% down to 7 μg/ml. This method was demonstrated to be useful in the presence of human growth hormone (M_r 22 000) but showed accuracy down to only 50 μg/ml. Use of an in-line protein A column (capable of binding IgG antibodies) with the size-exclusion column was shown to give similar detection limits in the presence of an intact antibody. © 1997 Elsevier Science B.V.

Keywords: Surfactants; Polysorbate 80; Proteins

1. Introduction

Proteins have become a common class of therapeutic agents with the advent of biotechnology. Formulations developed for these protein therapeutics differ widely depending on the nature of the protein, the route of administration and whether the product is a liquid or a lyophilized cake. Regardless of these differences, a common feature of many protein formulations is the presence of added surfactants. In liquid formulations, surfactants are added to minimize protein absorption to surfaces (containers and syringes) and to reduce the air–liquid interfacial surface tension in order to decrease the rate of protein denaturation that can lead to aggregation [1–3]. In lyophilized products, surfactants are frequently added to coat the air–solid interface of the lyophilized cake which is thought to protect the protein from surface denaturation [4].

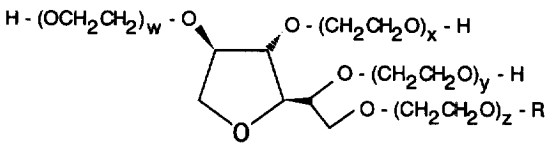
Surfactants fall into several categories based on their molecular charge: cationic, anionic, zwitterionic and nonionic. Nonionic surfactants are commonly used in the pharmaceutical industry due to minimal binding of the surfactant to the protein. Nonionic surfactants include polyoxyethylene sorbitans, polyoxyethylene ethers and polyethylene–polypropylene glycols.

In all pharmaceutical products, a quantitative

*Corresponding author.

0021-9673/97/$17.00 © 1997 Elsevier Science B.V. All rights reserved.
PII S0021-9673(97)00540-2

assay is used to verify the concentration of each component. However, some commonly used nonionic surfactants are extremely difficult to quantify in protein formulations because of their heterogeneous molecular composition. For example, polysorbate 80 has a chemical formula of



where R=oleate ($C_{18:1}$) and W+X+Y+Z=20. The number of possible molecular structures is greater than 1500. This heterogeneous mixture is not amenable to standard analytical techniques such as reversed-phase (RP) HPLC because of the complexity of the chromatogram especially in the presence of protein and excipients.

Commonly used quantitative methods for polysorbate 80 are quite time consuming and use environmentally hazardous solvents [5–9]. Typically, protein is precipitated out with a volatile organic solvent in which the surfactant is soluble. After separating and drying the solvent, the residual surfactant is resolubilized with a two-phase extraction system of aqueous cobalt and methylene chloride. The surfactant forms a complex with the cobalt and partitions into the organic phase. The concentration of complexed surfactant in the organic phase is then determined by optical absorption. Replacement of this laborious assay with a simple, fast, high precision method would be of great benefit.

This paper describes a single-step HPLC method that can be used to quantify surfactants in the presence of many excipients and, in some cases, proteins. The method consists of a size-exclusion chromatography (SEC) column utilizing a mobile phase containing surfactant at concentrations above the critical micelle concentration (CMC). The presence of micellar surfactant in the mobile phase shortens the elution time of the surfactant and disrupts any surfactant–column interactions thus sharpening the surfactant peak resolving it from the low-molecular-mass excipients. The method is demonstrated with polysorbate 80 in the absence and presence of protein.

2. Materials and methods

2.1. SEC methodology

2.1.1. Mobile phase preparation

The mobile phase buffer was 10 m*M* sodium phosphate in 150 m*M* sodium chloride, pH 7.0 (PBS). To make the 1000 μg/ml polysorbate 80 mobile phase, 10 ml of a 10% solution of polysorbate 80 (lot B0907, I.C.I. Chemical Polymers, Los Angeles, CA, USA) was added per liter of buffer solution. The mobile phase concentration of surfactant for each of the runs was made by mixing, on the HPLC system or manually, of the 1000 and 0 μg/ml surfactant mobile phases.

2.1.2. Preparation of samples

Samples for the surfactant standard curves were prepared by the serial dilution of PBS containing 1000 μg/ml surfactant with PBS containing no surfactant.

Gel filtration molecular mass standards (Bio-Rad, Hercules, CA, USA) were reconstituted with PBS containing no surfactant. Human growth hormone (hGH) lyophilized from ammonium bicarbonate was dissolved into PBS at a concentration of 5 mg/ml. Polysorbate 80 was added after dissolution and filtration through a 0.22 μm filter.

2.1.3. Instrumentation

All of the samples were analyzed on a 1090L HPLC system (Hewlett-Packard, Palo Alto, CA, USA) in triplicate utilizing a TSK G2000/SWXL size-exclusion column (TosoHaas, Japan, column size: 300 mm×7.8 mm; particle size: 5 μm). The method consisted of a 1 ml/min flow-rate, 12.5 min run time, using an absorption detector at 235 nm. The injection volume was 150 μl.

2.2. In-line protein A column and SEC methodology

2.2.1. Preparation of samples

Recombinant humanized anti-CD20 monoclonal antibody (Genentech, S. San Francisco, CA, USA) was formulated at 6 mg/ml in 25 m*M* citrate, 150 m*M* sodium chloride, pH 6.5. Antibody solutions

containing polysorbate 80 were made by the addition of an appropriate volume of 10% polysorbate 80 to this protein solution.

2.2.2. Instrumentation

Injections of anti-CD20 solutions with polysorbate 80, as described above, were analyzed on a 1090L HPLC system, in triplicate, utilizing an in-line protein A column and a TSK G2000/SWXL size-exclusion column. The protein A column was custom packed with a flow-rate of 1 ml/min with Protein A linked to CL-4B sepharose (Sigma, St. Louis, MO, USA) into a polyether ether ketone (PEEK) column (50 mm×4.6 mm) using a Poros Self Pack packing device (PerSeptive Biosystems, Framingham, MA, USA) as per manufacturer's instructions. The polysorbate antibody HPLC method used a mobile phase containing citrate buffer with 100 μg/ml polysorbate 80, 0.5 ml/min flow-rate, 45 min run time and a 50 μl injection. Data collection was performed as above.

3. Results and discussion

Surfactants, including polysorbate 80, can exhibit aggregation. At low concentrations in aqueous solution, surfactants exist as monomers. Above a characteristic limit, the CMC, micelles or clusters of surfactant molecules are formed. Micelle formation is a consequence of the amphipathic (hydrophobic–hydrophilic) nature of the surfactant. Since commercially available polysorbate 80 is a mixture of components it is necessary to determine the CMC for the exact lot used in this report. To determine the CMC for this lot of polysorbate 80, N-phenyl-1-naphthylamine (NPN), a fluorescent probe whose spectrum shifts in the presence of micelles, was utilized. Fig. 1 shows the fluorescence intensity of a constant concentration of NPN in varying concentrations of polysorbate 80. From this figure, the CMC of polysorbate 80 can be estimated as 7 μg/ml. Due to the heterogeneous nature of the surfactant there appears to be reproducible pre-micellar formation (~4 μg/ml) possibly comprised of longer or multiple fatty acid chain components. The molecules with longer or more fatty acid chains would be expected

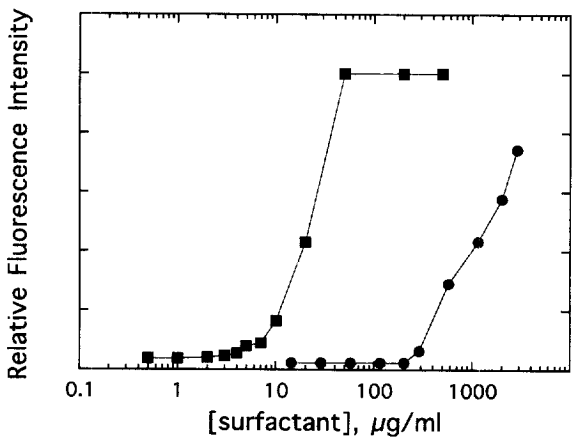


Fig. 1. Relative fluorescence intensity of NPN as a function of surfactant concentration. Solid squares (■) represent polysorbate 80, solid circles (●) represent SDS. An emission wavelength of 420 nm was monitored while utilizing an excitation wavelength of 350 nm. Concentration of NPN was 1 μ*M*.

to form micelles at lower concentrations. Except for the pre-micellar formation, the critical micelle concentration transition of polysorbate 80 is relatively sharp and compares well with that of sodium dodecyl sulfate (SDS), a single homogeneous surfactant. The consequence of a broad CMC will be discussed later.

In aqueous SEC, injection of a low concentration of surfactant results in a broad peak with a retention volume similar to a low-molecular-mass excipient. When the concentration of the injected surfactant is above the CMC, the injected micelles elute faster than the monomers with which they are in equilibrium. The faster eluting micelles dissociate to monomer to some extent resulting in a broad peak with a shorter retention time but an extended tail. The position of the peak and extent of the tail depends upon the injected concentration, the CMC and the dilution that occurs during elution through the column. An example of this effect is shown in Fig. 2 using polysorbate 80. As the injected surfactant concentration decreases so does the proportion of micelle to monomer and thus the resulting peak shifts to longer retention times. In addition, the chromatographic signals in Fig. 2 are normalized to the 1000 μg/ml surfactant injection and show a decrease in percent recovery with decreasing surfactant concentration. If there were no loss in recovery,

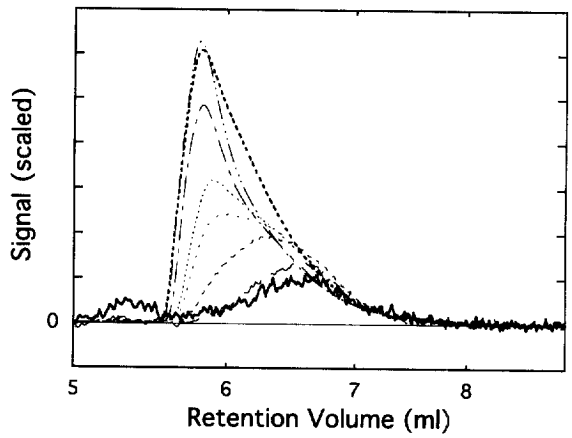


Fig. 2. Chromatograms of the polysorbate 80 standards with no polysorbate 80 in the mobile phase. Injected polysorbate 80 concentrations of 1000 (- - -), 500 (– · · ·), 200 (– · –), 100 (· · ·), 70 (- - -), 40 (– – –), 20 (— — —) and 10 (———) μg/ml. Chromatograms have been normalized to the 1000 μg/ml injection.

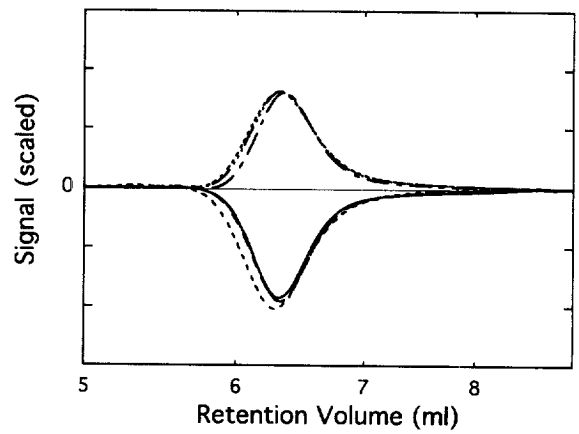


Fig. 3. Chromatograms of the polysorbate 80 standards with 100 μg/ml polysorbate 80 in the mobile phase. Injected polysorbate 80 concentrations of 1000 (- - -), 500 (– · · ·), 200 (– · –), 70 (- - -), 40 (– – –), 20 (— — —) and 10 (———) μg/ml. Chromatograms are normalized to the difference in the concentration injected from that of the mobile phase surfactant concentration.

the integrated area of the chromatograms would be the same.

The design of the method reported here introduces surfactant into the mobile phase to maintain the surfactant concentration above the CMC throughout elution through the column. Under such conditions, injection of surfactant at concentrations above that found in the mobile phase will result in a positive peak that has a retention volume of the micelles. Injections of surfactant concentrations below that of the mobile phase will result in a valley (negative peak) with a similar retention volume and width. An example using polysorbate 80 is displayed in Fig. 3. In this figure the chromatograms are normalized to the difference in the concentration injected from that of the mobile phase surfactant concentration. It is evident that with surfactant in the mobile phase, the percent recovery is constant throughout the range tested and there was no substantial peak position changes with injected surfactant concentration.

Fig. 4 shows a plot of the area under the curve (AUC) of polysorbate 80 standards at four mobile phase surfactant concentrations. In the absence of surfactant in the mobile phase, the standard curve is not linear at the concentrations tested. This is likely due to the dissociation of micellar polysorbate 80 as it elutes through the column. The injected surfactant dilutes by approximately a factor of 4 (150 μl injected, 600 μl full width at half peak height). In the presence of surfactant in the mobile phase the data is linear and the slope of the linear fit is the same for each of the mobile phase concentrations. In addition, the x-intercept of the best fit line for each of the mobile phases is very close to the estimated surfactant concentration in the mobile phase. This indicates that there is no significant loss of recovery when the surfactant is present in the mobile phase as compared to no surfactant in the mobile phase. As shown in the insert in Fig. 4 the calculated y-intercepts of each of the standards are linear with mobile phase surfactant concentration and intercepts the origin. This suggests that there is no loss of surfactant recovery at the concentrations tested.

The lower concentration limit of this assay was studied at a mobile phase surfactant concentration of 20 μg/ml polysorbate 80. This concentration is slightly above the CMC (7 μg/ml) as shown in Fig. 1. The results are shown in Fig. 5 where a linear fit was observed down to an injected surfactant concentration of 2 μg/ml at which point the response was the same as injection of buffer alone. From replicate injections, surfactant concentrations above 7 μg/ml can be determined with less than a 10% R.S.D.. Injection of surfactant in the absence of

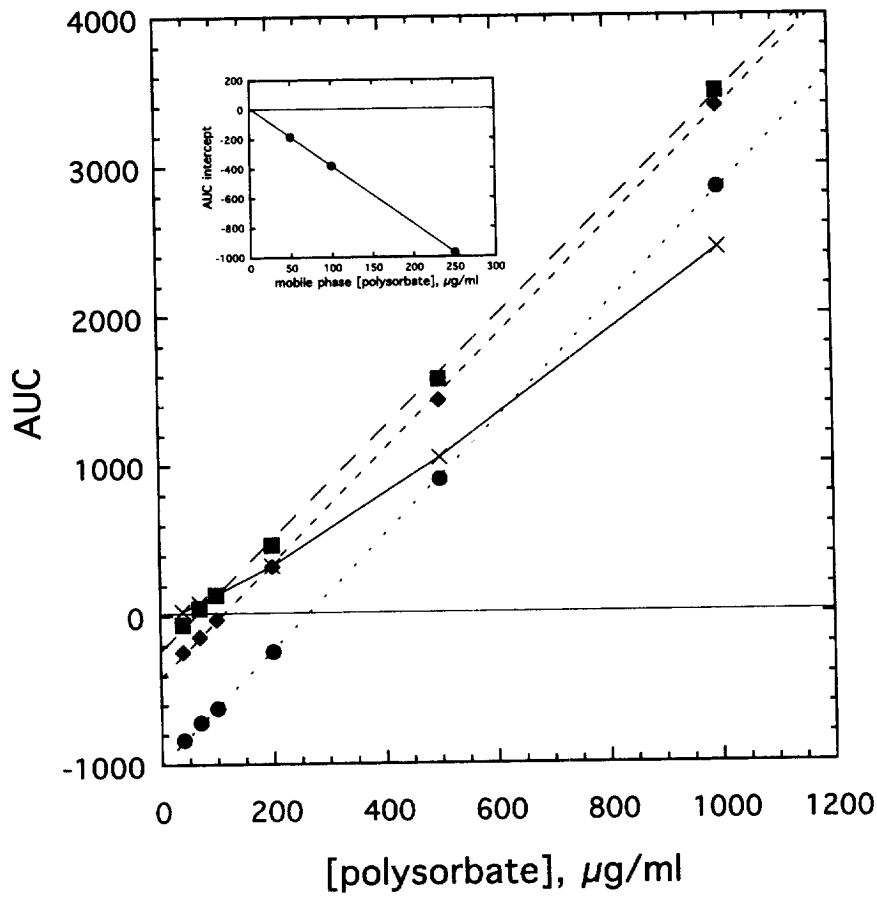


Fig. 4. Plot of the area under the curve (AUC) for the polysorbate standards. A mobile phase of PBS containing 0 (×), 50 (■), 100 (♦), 250 (●) μg/ml polysorbate 80 was used. Inset depicts the *y*-intercept, as extrapolated from the main graph, as a function of mobile phase polysorbate concentration.

surfactant in the mobile phase is undetectable at concentrations below 10–20 μg/ml. This demonstrates that the presence of low concentrations of surfactant in the mobile phase can increase recovery and sensitivity of the assay.

Fig. 6 demonstrates that the presence of surfactants in the mobile phase does not affect the general dispersive characteristics of the column. Neither significant peak broadening nor shifting of the molecular mass standards were observed in the presence of surfactant in the mobile phase. The observed trough is a result of injecting the molecular mass standard solution which contained less surfactant than the mobile phase. The proteins that elute before and after the trough are in equilibrium with the mobile phase surfactant concentration. Fig. 6 demonstrates the potential usefulness of the method in the presence of proteins of molecular sizes that are, in general, smaller or larger than that of the micellar surfactant. By use of a column with the proper dispersive characteristics, this method can be used to quantify polysorbate 80 concentrations in the presence of a variety of sizes of proteins. As an example, hGH (M_r 22 000) was prepared in PBS with 50, 100, 200 and 475 μg/ml polysorbate 80. Chromatographic analysis of the above samples using 100 μg/ml polysorbate 80 in the mobile phase resulted in observed values of 51, 84, 182 and 466 μg/ml polysorbate 80, respectively. This demonstrates that, in the presence of protein, polysorbate

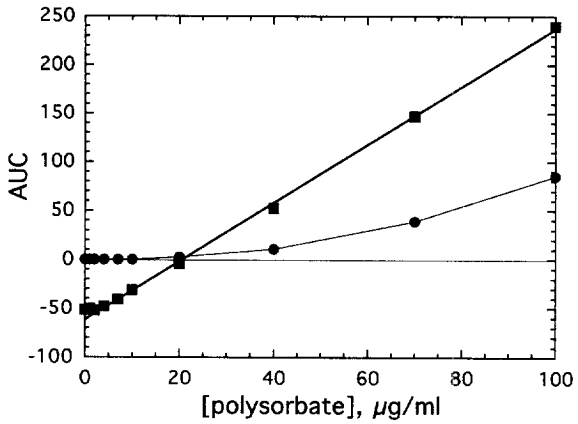


Fig. 5. Plot of the area under the curve (AUC) for the low concentration polysorbate standards. A mobile phase of PBS containing 0 (■) and 20 (●) μg/ml polysorbate 80 was used.

concentrations can be determined relatively accurately. To maximize the polysorbate 80 signal over the protein signal (5 mg/ml hGH), detection at 247 nm was used where the ratio of protein optical absorption to polysorbate 80 optical absorption is at a minimum.

Unfortunately, a size-exclusion column alone may not be sufficient to resolve the protein from the micellar surfactant peak for other proteins or surfactants. It is possible that a pre-column could be used to capture or prolong the retention of the protein to avoid co-elution of the protein with the micellar surfactant. A wide array of pre-columns could be used utilizing a variety of chromatographic properties. For instance, a heparin column could be used to bind specific heparin binding proteins such as basic fibroblast growth factor or vascular endothelial growth factor. In a similar fashion, an anion-exchange column could be used for acidic proteins or a cation-exchange column could be used for basic proteins to bind the interfering protein or prolong its retention. In most cases, the pre-column, upon equilibration with the mobile phase, should not affect the dispersive properties of the size-exclusion column or the micellar properties of the surfactant. Results of a test of a true pharmaceutical formulation using an in-line protein A column to bind an IgG antibody is shown in Fig. 7. Recombinant humanized anti-CD20 antibody (M_r 150 000) was prepared in isotonic citrate buffer with 50, 100, 500 and 700 μg/ml polysorbate 80. The latter formulation is currently in clinical trials for treatment of follicular

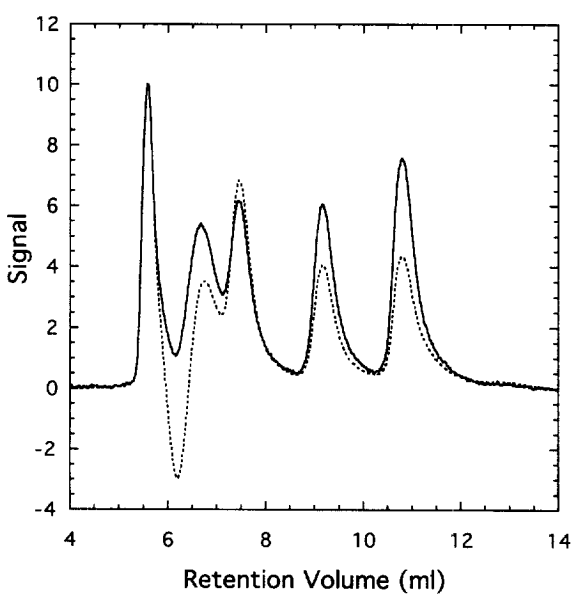


Fig. 6. Plot of chromatograms of the gel filtration molecular mass standards. A mobile phase of PBS with (· · ·) and without (——) 500 μg/ml polysorbate 80 was utilized. Molecular mass standards are comprised of thyroglobulin (M_r 670 000), γ-globulin (M_r 158 000), ovalbumin (M_r 44 000), myoglobin (M_r 17 000) and cyanocobalamine (M_r 1300).

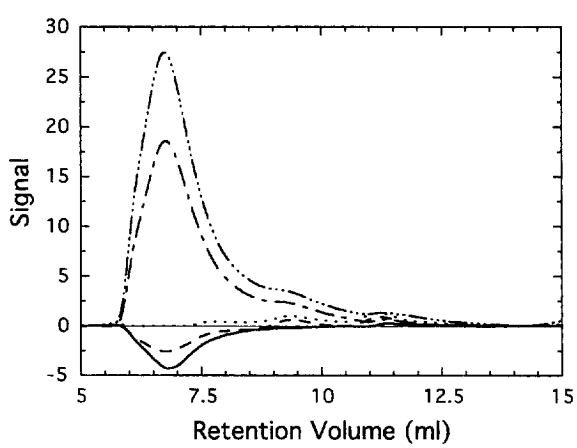


Fig. 7. Plot of chromatograms of anti-CD20 IgG containing polysorbate 80. Injected solutions contained 6 mg/ml anti-CD20 in isotonic citrate with 0 (——), 50 (— — —), 100 (· · ·), 500 (–·–) and 700 (– · · ·) μg/ml polysorbate 80 and the mobile phase contained 100 μg/ml polysorbate 80 in isotonic citrate.

non-Hodgkin's lymphoma. Chromatographic analysis by peak heights of the above samples using 100 μg/ml polysorbate 80 in the mobile phase resulted in 43, 97, 518 and 717 μg/ml polysorbate 80, respectively. Peak heights were used in the analysis of these formulations because of the difficulty in integration of the surfactant peak due to the presence of a small but interfering peak from the protein preparation. Further use of an in-line column to capture interfering protein is being studied in this laboratory.

As with any method there are limitations. The protein may bind several equivalents of surfactant. This would be more likely with cationic or anionic surfactants where hydrophobic and electrostatic interactions could lead to such binding. In such circumstances, the apparent surfactant concentration of the sample would be lower than the actual concentration. In fact, injection of a protein solution with no surfactant onto a sizing column with surfactant in the mobile phase will result in a micellar valley deeper than that of buffer alone. This effect has been described by Hummel and Dreyer [10] and can be used to quantify the amount of binding of the surfactant to the protein. The extent of this effect would have to be determined empirically for each protein and surfactant concentration. For the proteins tested in this study, negligible binding was observed with polysorbate 80 (a non-ionic surfactant). However, under the worst of conditions it may be necessary to precipitate the protein and extract the surfactant with a volatile organic solvent. The solvent could then be dried and the surfactant resolubilized and assayed by this method. This would make the two-phase extraction and colorimetric determination of the standard surfactant assay, as described in Section 1, unnecessary. Alternatively, a solution with the appropriate protein concentration could be used to make up the surfactant concentration standards.

The use of surfactants that have high CMCs may also be problematic. If the surfactant concentration of the tested sample is low but the mobile phase concentration must be high to remain above the CMC, the error in the AUC determination may be too great to obtain meaningful data. With surfactants that exhibit a broad CMC, the surfactant peak may be too broad to accurately quantify. These limitations may be overcome to some extent by adjustments to the mobile phase ionic strength or temperature to lower the CMC.

Formulations that contain two different surfactants may be difficult to analyze due to the formation of mixed micelles. Depending upon the amounts of each of the surfactant components, micelles of various sizes and thus various retention times would be expected. Even with a fixed concentration of surfactant(s) in the mobile phase, differences in micelle size would be expected with different concentrations of injected surfactants. The resulting shift in retention time and the possible formation of multiple peaks would hamper quantitation of the surfactant of interest.

4. Conclusions

A simple HPLC method is described that has the potential of determining the concentration of numerous classes of surfactants in the presence of protein and excipients. This method can also be adapted for use with other in-line columns and detectors if necessary. Optimization of the method for other surfactants may require 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.

Acknowledgments

The authors would like to thank Ms. Janet Yang and Ms. Xanthe Lam for their technical assistance, Ms. Eileen T. Duenas and Ms. Mary E.M. Cromwell for their useful discussions, and Ms. Anita R. Ojha and Ms. Sabrina Kivitz for their help in buffer preparation.

References

[1] A.S. Chawla, I. Hinberg, P. Blais, D. Johnson, Diabetes 34 (1985) 420–424.
[2] W.D. Loughheed, A.M. Albisser, H.M. Martindale, J.C. Chow, J.R. Clement, Diabetes 32 (1983) 424–432.
[3] Z.J. Twardowski, K.D. Nolph, T.J. McGray, H.L. Moore, Am. J. Hosp. Pharm. 40 (1983) 579–581.

[4] C.C. Hsu, H.M. Nguyen, D.A. Yeung, D.A. Brooks, G.S. Koe, T.A. Bewley, R. Pearlman, Pharm. Res. 12 (1995) 69–77.
[5] E.G. Brown, T.J. Hayes, Analyst 80 (1955) 755–767.
[6] N.T. Crabb, H.E. Persinger, J. Am. Oil Chem. Soc. 41 (1964) 752–755.
[7] R.A. Greff, E.A. Setzkorn, W.D. Leslie, J. Am. Oil Chem. Soc. 42 (1965) 180–185.
[8] A. Nozawa, T. Ohnuma, S. Tatsuya, Analyst 101 (1976) 543–548.
[9] N.H. Anderson, J. Girling, Analyst 107 (1982) 836–838.
[10] J.P. Hummel, W.J. Dreyer, Biochim. Biophys. Acta 63 (1962) 532–534.