Analytical Development[1], Bayer Schering Pharma AG, Berlin; Department of Biopharmaceutics and Pharmaceutical Technology[2], Ernst-Moritz-Arndt-Universität, Greifswald, Germany

Stability of the non-ionic surfactant polysorbate 80 investigated by HPLC-MS and charged aerosol detector

A. Christiansen[1,2], T. Backensfeld[1], S. Kühn[1], W. Weitschies[2]

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Prof. Dr. Werner Weitschies, Department of Biopharmaceutics and Pharmaceutical Technology, Ernst-Moritz-Arndt-Universität Greifswald, Friedrich-Ludwig-Jahn-Str. 17, 17487 Greifswald, Germany
werner.weitschies@uni-greifswald.de

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An analytical method using HPLC coupled with a charged aerosol detector (CAD) and a mass selective detector (MSD) was developed to characterize the non-ionic surfactant polysorbate 80 (PS 80). The molecular structure and heterogeneous composition due to isomers and various lengths of PEG-chains make it difficult to develop sensitive and specific analytical methods. Hence, there is only limited knowledge about the stability and purity of this compound. Polysorbate 80 does not possess any chromophore, thus UV detection is not applicable. Therefore, CAD and MSD have been used for determination. The aim of this study was to characterize polysorbate 80 and to examine its stability at pH 1.0 and 37 °C simulating harsh gastric conditions. It was shown that this surfactant is liable to degradation under these conditions. Within 8 h monoesters of PS 80 were hydrolyzed to an extent of 9.5% (±3.0%), whereas incubation in water did not result in any detectable degradation. Furthermore, we demonstrated that HPLC-MS is a suitable technique to investigate ethoxylated compounds like polysorbates.

1. Introduction

Amphiphilic excipients are commonly applied in order to improve the water solubility of drug substances and thereby enhance their bioavailability (Strickley 2004).This is of increasing relevance as many of the new drugs show poor aqueous solubility which is associated with limited oral bioavailability (Lipinski 2000). Meanwhile it is increasingly recognized that amphiphilic excipients may affect the bioavailability of drugs not only by increasing their water solubility by direct solubilization, but also by alteration of the composition of the intestinal milieu which may result in an alteration of its solubilizing properties (Porter et al. 2007). In recent years much attention had been focused on lipid-based formulations (e.g. lipid suspensions, solutions and emulsions) with particular emphasis on self-emulsifying drug delivery systems (SEDDS) which are isotropic mixtures of oils, surfactants, solvents and co-solvents/surfactants (Gursoy and Benita 2004). These lipidic formulations and their digestion products form a range of vesicular and micellar species with endogenous bile salts and phospholipids which leads to an enhanced solubilization capacity for the API in the intestinal lumen (Porter et al. 2007). In any case, formulations of Type II-IV according to the Lipid Formulation Classification System (LFCS) contain high amounts of non-ionic surfactants (Pouton 2006). Moreover, certain surfactants interfere with cytochrome (CYP) P450 enzymes which primarily metabolize many hydrophobic drugs, whereas inhibitory interactions can diminish their degradation (Mountfield et al. 2000; Ren et al. 2008). In addition, it was shown *in vitro* and *in vivo* that inhibition of P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP) by certain non-ionic surfactants can lead to an increased absorption rate (Cornaire et al. 2004; Schulze et al. 2005).

Excipients are historically considered to be pharmacologically inert and show no effects on transporters or enzymes. Hence, for a long time formulation developers had focused exclusively on their technological functions such as drug solubilization in case of surfactants and did not take much account of stability and potential interactions which also might be assigned to degradation products. However, in case of amphiphilic excipients there is insufficient knowledge about their influence on metabolic enzymes and transporter systems after oral administration. Particularly, a critical issue is that there is currently also a lack of studies on their stability under physiologically conditions. Quality specifications of excipients often differ in their quality requirements for identical compounds although some global standardized monographs exist (Baldrick 2000). The analytical parameters defined are often not relevant for formulation development and do not reflect stability indicating criteria. These aspects highlight the necessity of attracting notice to the fate of excipients in the gastrointestinal tract after oral administration. Therefore, analytical methods are required to test their stability under physiological conditions as well as in presence of digestive enzymes. The identification of degradation products should also be an objective regarding possible effects on transporters and/or enzymes as well as an influence on the API solubilization. Among others polysorbate 80 (PS 80) is a commonly used non-ionic surfactant. Polysorbate 80 is an oleate ester of sorbitol and its anhydrides copolymerized with approximately 20 mol polyethylene glycol (PEG) for each mol of sorbitol and sorbitol anhydrides (Fig. 1) (US Pharmacopeia XXXII) and therefore an example of a polyethoxylated surfactant. Polysorbate 80 is employed in lipid-based formulations, e.g. Rapamune® (Sirolimus) or Gengraf® (Cyclosporin), like other polyethoxylated surfactants such as polysorbate 20 and diverse types of Cremophors. The molecular structures of this surfactant and its

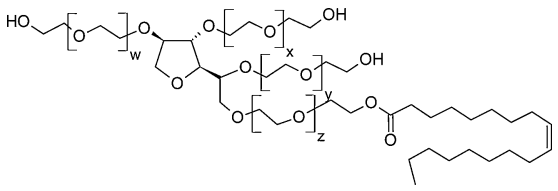

Fig. 1: Molecular structure of polysorbate 80 (w + x + y + z = 20)

heterogeneous composition due to isomers, various lengths of PEG-chains, and impurities make it difficult and complex to develop sensitive and specific analytical methods. Hence, there is marginal literature about data concerning stability, composition and purity of polysorbate 80.

For quantification it has been suggested to perform derivatization in order to transform the non-absorbing into a chromophoric compound which can be analysed using UV detection. Heinig et al. (1998) reported derivatization with phenyl isocyanate. A further method for the quantification of polysorbate 80 developed by Hu et al. (2003) requires saponification with dilute potassium hydroxide and subsequent determination of the released oleic acid with HPLC-UV at 210 nm. The European Pharmacopeia 6.6 recommends the determination of the composition of the fatty acid fraction after saponification as well (European Pharmacopoeia, 2010). However, with knowledge of the heterogeneity of this surfactant and the presence of impurities, it is obvious that these methods are not sufficient for an exact quantification and characterization of this complex excipient.

The purpose of this study was to develop a suitable HPLC method to characterize polysorbate 80 and to investigate the degradation at pH 1.0 and 37 °C simulating harsh gastric conditions. Due to the lack of chromophores analyses were performed with HPLC coupled to a charged aerosol detector (CAD) and a mass selective detector (MSD). The CAD generates a universal response for non-volatile compounds by detecting charged aerosol particles and measuring the current from the charged particle flux.

2. Investigations and results

In Fig. 2 a chromatogram as acquired after injection of 1 µg polysorbate 80 (PS 80) obtained with the CAD is shown. Three groups of peaks can be identified which are eluted at different percentages of ACN in the mobile phase. They are derived from three groups of structurally similar compounds which cannot be completely separated. At the beginning non-esterified hydrophilic components are eluted. At higher percentage of organic solvent first mono-esterified (60% ACN) followed by more lipophilic di-esterified PEG sorbitans (93% ACN) are eluted. Fig. 3 illustrates the heterogeneity of components which are eluted at a percentage of 60% ACN. Overlaying series of masses with the difference of one PEG monomer (*m/z* 44) are revealed (*m/z* 808, 852, 896, 940, 984, 1028 and 869, 913, 957, 1001). Table 1 shows theoretically possible structures of the main serial which represent the detected molecular weights (MW) of high intensity. Setting up these masses, chromatograms of 2.5 µg PS 80 in the selected ion mode (SIM) were measured with the MSD (Fig. 4). The main compounds are eluted with a percentage of 60% ACN in the mobile phase which can be related to polyethylene glycol isosorbide monooleates with the number of 9–14 PEG monomers. Small amounts of hydrophilic PEG isosorbides with 15–20 PEG monomers and PEG isosorbide dioleates with 3–8 PEG monomers are also detected in the sample. The second serial (m/z 869, 913, 957, 1001) which is eluted with a percentage of 60% ACN is obtained from PEG sorbitan monooleates with the number of 10–13 PEG monomers (Table 2).

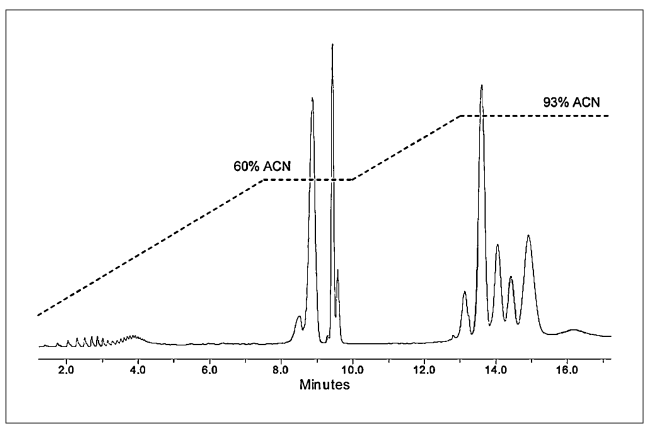

Fig. 2: Chromatogram obtained with the CAD of 1 µg polysorbate 80 dissolved in water

Table 1: Theoretically possible structures which possess the molecular weights (MW) of high intensity in the mass spectrum of polysorbate 80

$MW + H^+$	compound
807	PEG (9) isosorbide monooleate
851	PEG (10) isosorbide monooleate
897	PEG (11) isosorbide monooleate
939	PEG (12) isosorbide monooleate
983	PEG (13) isosorbide monooleate
1027	PEG (14) isosorbide monooleate
807	PEG (3) isosorbide dioleate
851	PEG (4) isosorbide dioleate
897	PEG (5) isosorbide dioleate
939	PEG (6) isosorbide dioleate
983	PEG (7) isosorbide dioleate
1027	PEG (8) isosorbide dioleate
807	PEG (15) isosorbide
851	PEG (16) isosorbide
897	PEG (17) isosorbide
939	PEG (18) isosorbide
983	PEG (19) isosorbide
1027	PEG (20) isosorbide

Table 2: Theoretically possible structures which possess the molecular weights (MW) of the second serial with lower intensity in the mass spectrum of polysorbate 80

MW	compound
869	PEG (10) sorbitan monooleate
913	PEG (11) sorbitan monooleate
957	PEG (12) sorbitan monooleate
1001	PEG (13) sorbitan monooleate
869	PEG (4) sorbitan dioleate
913	PEG (5) sorbitan dioleate
957	PEG (6) sorbitan dioleate
1001	PEG (7) sorbitan dioleate
869	PEG (16) sorbitan
913	PEG (17) sorbitan
957	PEG (18) sorbitan
1001	PEG (19) sorbitan

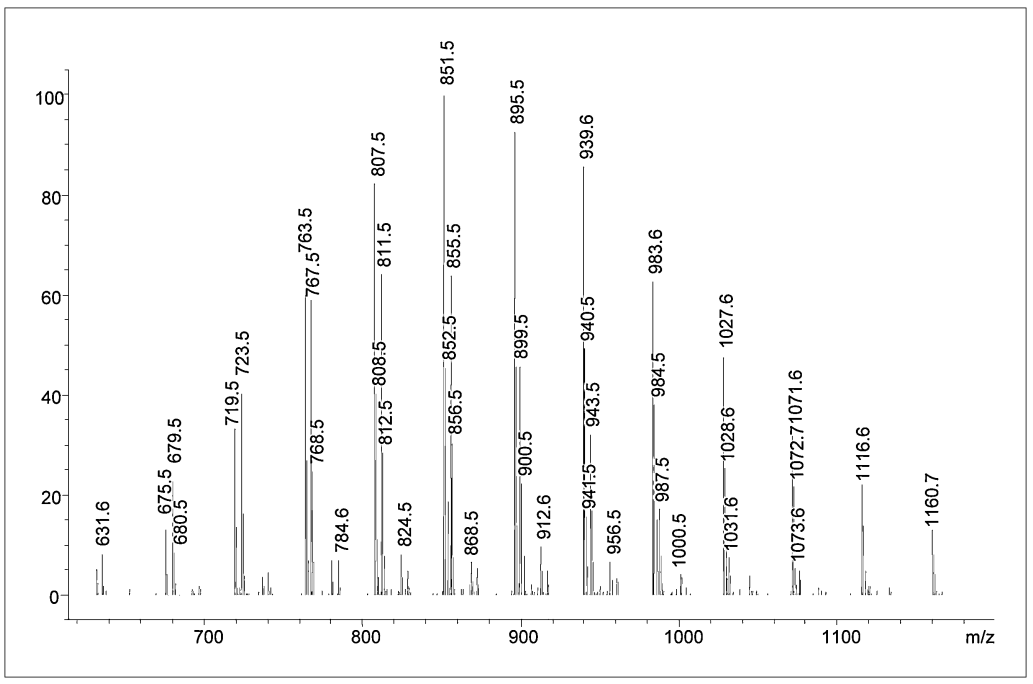

Fig. 3: Mass spectral data of the peak group of polysorbate 80 monoesters eluted at a percentage of 60% ACN: The protonated ions were detected which then are one mass unit higher than the molecular weight of the original structures. Therefore, they correspond to the main compounds with the MW of 675, 719, 763, 807, 851, 897, 939, 1027, 1071, 1115 g/mol which are PEG isosorbide monoesters with the number of 6–16 PEG monomers. The second serial of masses with high intensity is derived from protonated PEG monooleates with the number of 9–17 PEG monomers (*m/z* 680, 724, 768, 812, 856, 900, 944, 988, 1032). Ions are observed in two series: the ions of one series have exclusively ^{12}C carbon isotopes, and ions of the second series show one ^{13}C carbon isotope

From the measurement of different amounts of polysorbate 80 a calibration curve generated from 5 points could be plotted in the range of 0.01–0.07 mg/ml, which provided linearity with a correlation coefficient of 0.9998 (data not shown). Repeatability (5 injections) resulted in a variation coefficient of 2.6% (2.5 µg PS 80) and 3.6% (5 µg PS 80). For all tests water had been used for preparing the standard solutions.

With this analytical method the degradation of polysorbate 80 in HCl pH 1.0 at 37 °C was measured in comparison to incubation in water (Fig. 5). PEG (9–14) isosorbide monooleate, which was found prevalently in the sample, had been hydrolyzed to an extent of 9.5% (± 3.0%) within 8 h. Within 4 h minor decomposition of 3.8% (± 2.7%) proceeded. Incubation of PS 80 in water did not result in any detectable degradation.

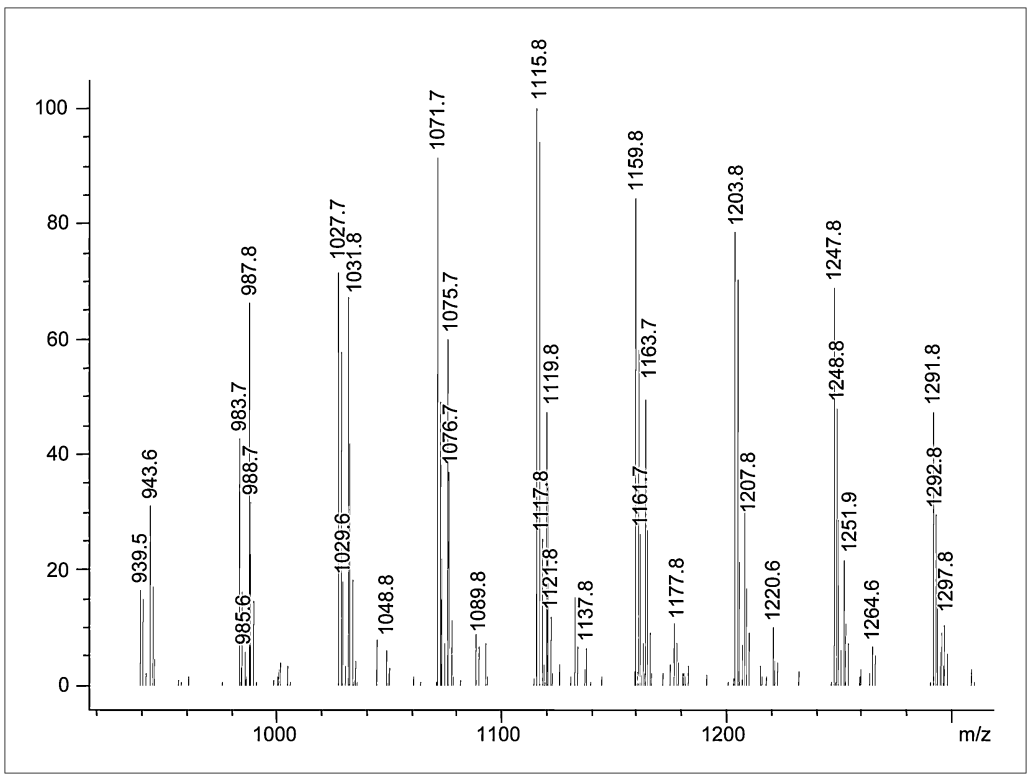

Fig. 4: Mass spectral data of the peak group of polysorbate 80 diesters eluted at a percentage of 93% ACN: Mainly PEG isosorbide diesters the number of 6–14 PEG monomers are detected (*m/z* 940, 984, 1028, 1072, 1116, 1160, 1204, 1248, 1292). A second serial of ions is derived from PEG dioleates with the number of PEG monomers of 9–16 (*m/z* 944, 988, 1032, 1076, 1120, 1164, 1208, 1252)

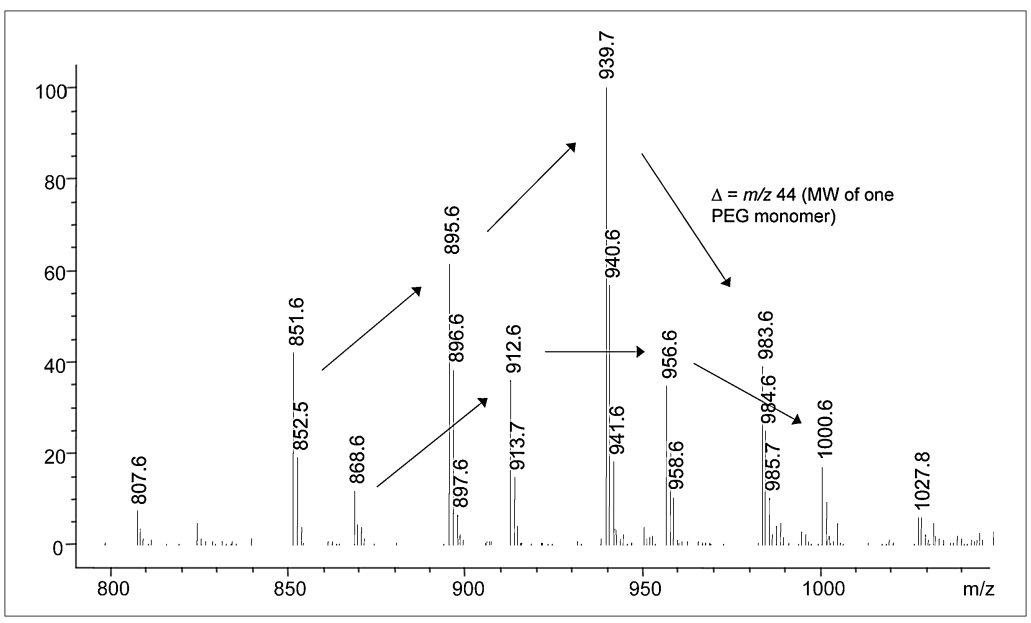

Fig. 5: Mass spectral data of the main peak in the CAD chromatogram of polysorbate 80 monoesters at t = 9.3 min: The detected masses originate from PEG monooleates with the number of 9–14 PEG monomers (*m/z* 808, 852, 896, 940, 1028). The second serial (m/z 869, 913, 957, 1001) is obtained from protonated PEG sorbitan monooleates with the number of 10–13 PEG monomers (*m/z* 869, 913, 957, 1001)

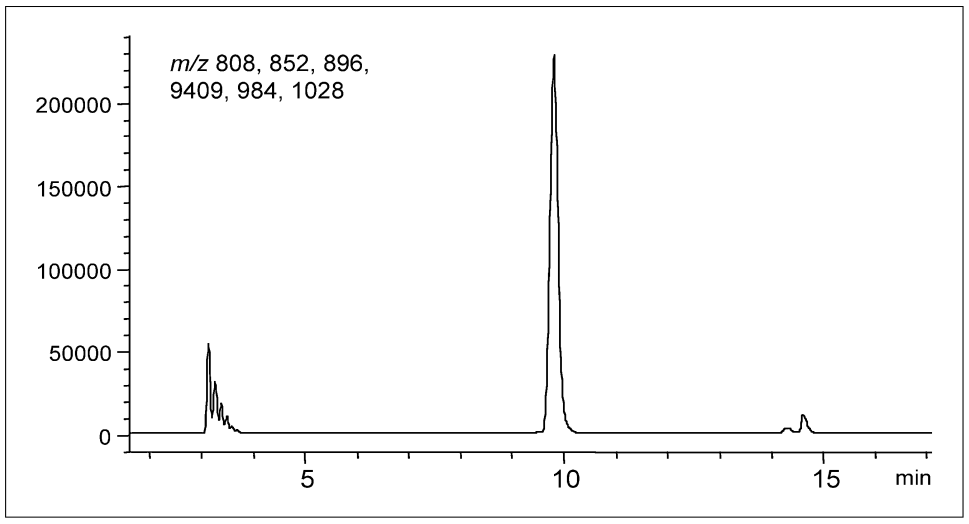

Fig. 6: Chromatogram of polysorbate 80 obtained in the selected ion mode (SIM) analyzing the second peak of monooleates: *m/z* 808, 852, 896, 940 and 1028

3. Discussion

In this study the ultra-pure Polysorbate 80 (HX)™ manufactured by NOF Corporation (Tokyo, Japan) was used with an oleic acid content of more than 99%. It is specified to be highly stable, colourless and odourless, and to contain low amounts of impurities due to an upgraded ethoxylation technology and purification technique of oleic acid (NOF Corporation, 2005). The European Pharmacopoeia 6.6 requires for polysorbate 80 a minimum content of 58.0% of oleic acid and certain amounts of other fatty acids (e.g. maximum 18% linoleic and 16% palmitic acid) are tolerated (European Pharmacopoeia, 2010). However, it is revealed in the chromatogram (Fig. 2) that the purified product also shows a quite heterogeneous composition. This is in good agreement with previous observations. Ayorinde et al. obtained by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry that polysorbate 80 is a complex mixture of oligomers including polyethylene glycol (PEG), sorbitan polyethoxylates, isosorbide polyethoxylates, sorbitan polyethoxylate mono- and diesters, and isosorbide polyethoxylate mono- and diesters, all

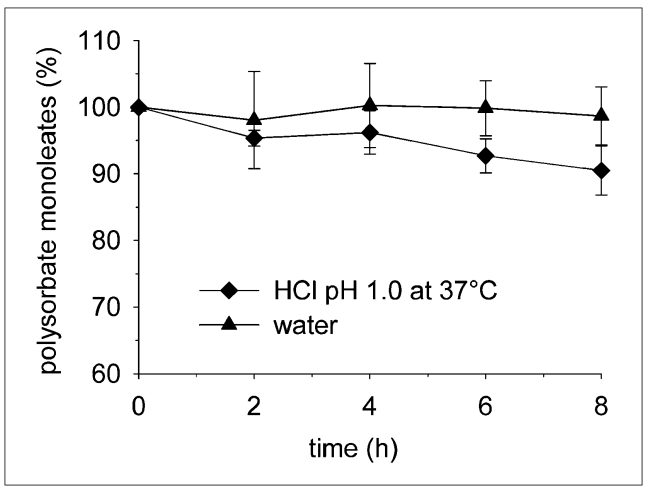

Fig. 7: Degradation of polysorbate monoesters in HCl Ph 1.0 at 37 °C (mean ± SD, $n = 6$). Concentration of the test solution was 0.05 mg/ml

of them with different lengths of PEG-chains. Thus, not all species contain a fatty acid at all. The complexities of the spectra are related to the fatty acid composition that was used in the manufacturing process (Ayorinde et al. 2000; Frison-Norrie and Sporns 2001). Other studies yielded that the same products differ between suppliers and even from batch to batch in terms of proportions of different fatty acids, sorbitol species, and amount of PEG mono-, di- and non-esterified components (Raith et al. 2006; Vu Dang et al. 2006). Wuelfing et al. (2006) investigated UV/Vis spectral characteristics for a series of commercially available sources of polysorbate 80. Theoretically it is a fully saturated molecule with only one carbon-carbon double bond, an alkyl ester group, and polyethylene glycol chains, accordingly absorbance at wavelengths not exceeding 210 nm should be expected. They suggest that absorption at higher wavelengths (220–340 nm), which appear with different molar absorptivity constants (ε) in all tested products from different vendors, are attributable to impurities. Only the spectra of the purified product from NOF Corporation nearly show the proposed characteristics (Wuelfing et al. 2006). Therefore, it is likely that the higher wavelength absorbance results from extended π-conjugation in fatty acid impurities. For this reason UV detection is precluded when investigating polysorbate 80.

In case of using HPLC-CAD for analysis, components are roughly separated by their different grades of esterification, but total separation and identification is not feasible with this universal detection method (Fig. 2). Furthermore, quantification is critical when a gradient is used as mobile phase. A CAD possesses a response which is independent of chemical properties of the analytes, but it depends on the percentage of organic solvent in the mobile phase due to changes in viscosity and therefore droplet size (Gamache et al. 2005). Accordingly, the amount of diesters in the sample might be overestimated and cannot be compared to the amount of monoesters.

However, the degradation of polysorbate 80 does not appear to be significant, since the gastric residence period is usually shorter than 8 h. In case of single unit dosage forms it also depends on the food intake. Compared to the fasting state, the gastric residence of solids is prolonged in the fed state and is also controlled by mass and quality of the food (Stotzer and Abrahamsson 2000). Length of time uncommonly exceeds 4 h and rarely reaches 8 h, furthermore in case of fluids and smaller particles the gastric residence is much shorter (Davis et al. 1986).

Here, we employed chromatograms that were obtained in the selected ion mode (SIM) of the lead structures of heterogeneous compounds like polyethoxylated surfactants for the quantification of decomposition. Prerequisite is prior identification of the lead structures of the ethoxylated compound when applying this method. Therefore, total ion chromatograms (TIC) of every product from different vendors or even every batch have to be recorded in order to discover the compounds which prevalently occur in the sample.

In conclusion with this work we have been able to propose an analytical method for quality control and stability testing, which allow a specific detection of the degradation of the non-ionic surfactant polysorbate 80. Moreover, we presume that the developed method can be used for the stability testing of this excipient in the presence of metabolic and digestive enzymes and might therefore be used to assess the potential for interactions with metabolic enzymes and transporters in the gastrointestinal tract. Applying this method during formulation development of SEDDS might disclose stability issues and thus support identification of relevant formulation parameters. Furthermore, the developed MS detection method can be assigned to other ethoxylated surfactants, respectively other species of polysorbate like Tween® 20.

4. Experimental

4.1. Chemicals and reagents

Polysorbate 80 (HX)™ was purchased from NOF Corporations (Tokyo, Japan). Acetonitrile (ACN) gradient grade was purchased from Merck (Darmstadt, Germany). The HPLC grade water was prepared from deionized water with a Millipore system (Milli-Q).

4.2. Instrumentation and parameters

The chromatographic system was a HP 1100 series from Agilent Technologies (Waldbronn, Germany) equipped with degasser, binary gradient pump, and tempered autosampler. It was controlled by ChemStation™ software. Moreover it was coupled to a charged aerosol detector (Corona™ CAD) from ESA Biosciences (Chelmsford, USA) and a single quadruopole mass selective detector (MSD), which also belongs to the HP 1100 series from Agilent Technologies. The ionization mode was atmospheric pressure chemical ionization (APCI) and analysis was performed in the positive ion mode. Gas temperature was set to 250 °C and the vaporizer had a temperature of 300 °C. Drying gas flow rate was 12.0 l/min and nebulizing gas pressure was set to 35 psi. Capillary voltage was 4200 V, corona current was set to 5.0 µA, and the optimal fragmentor voltage was 140 V. For quantification chromatograms were acquired in the selected ion mode (SIM): *m/z* 808, 852, 896, 940, 984 and 1028, because these ions appear to be the protonated compounds which prevalently occur in the sample. The CAD parameters were set to the nitrogen pressure of 35 psi and the sensitivity of 100 pA.

4.3. HPLC conditions

Separation was carried out on a Zorbax SB-Aq C18 (3.5 µm; 2.1 × 100 mm) from Agilent Technologies (Waldbronn, Germany). Flow rate and temperature were in case of detection with the CAD 1.0 ml/min and 50 °C. With use of the MSD it was set to 0.8 ml/min at 35 °C. An acetonitrile-water gradient was used as mobile phase increasing linearly from 15% ACN to 60% within 7.5 min, staying isocratic for 2.5 min and increasing again up to 93% ACN within 3 min, where it was kept isocratic for 6 min.

4.4. Sample preparation

By means of an ultrasonic bath polysorbate 80 was solved in water and hydrochloric acid solution (pH 1.0) for the incubation studies, respectively. In the incubation study the resulting concentration was 0.05 mg/ml.

4.5. Incubation studies

The sample solutions were incubated in tempered glass vessels at 37 °C for 8 h and under stirring. At the beginning of the incubation and after 2, 4, 6 and 8 h samples were taken and transferred into HPLC vials for analysis. 50 µl of the sample were injected into the HPLC.

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