SUMMARY

Surprisingly, it was found that a concrete marker substance, namely polyethylene glycol-6-laurate (PEG-6-laurate), is linked to the oxidative degradation route of polysorbate 20, because this marker substance is formed during oxidative degradation of polysorbate 20. This marker substance allows one to determine the degree of oxidative degradation of polysorbate 20 in aqueous formulations by an analytical method, since the relative level of said marker substance indicates that an oxidative degradation has taken place.

Therefore, it is provided a (first) method (in a first alternative) for determining the degree of oxidative degradation of polysorbate 20 in an aqueous formulation containing polysorbate 20, said method comprising the following steps:

step 1 providing a first sample of the aqueous formulation to be examined, which serves as a reference sample;

step 2 providing a second sample and optionally further samples of the aqueous formulation to be examined;

step 3 using a liquid chromatography-mass spectrometry based method for separating polyethylene glycol-6-laurate and determining the peak area of polyethylene glycol-6-laurate, the oxidation marker for the oxidative degradation of polysorbate 20, from the reference sample of step 1, and from the second sample and each optional further sample of step 2, respectively, and
determining the relative peak area of polyethylene glycol-6-laurate based on the peak area of polyethylene glycol-6-laurate in the second and each optional further sample in relation to the peak area of polyethylene glycol-6-laurate in the reference sample, the relative peak area being proportional to the degree of oxidative degradation of polysorbate 20; and

step 4 optionally quantifying the amount of polyethylene glycol-6-laurate in the second sample and each optional further sample based on the relative peak area of the respective sample.

In one aspect, the present disclosure provides a method for determining the degree of oxidative degradation of polysorbate 20 in an aqueous formulation containing polysorbate 20, said method comprising the following steps:

step 1 providing a first sample of the aqueous formulation to be examined, which serves as a reference sample;

step 2 providing a second sample and optionally further samples of the aqueous formulation to be examined;

step 3 using a liquid chromatography-mass spectrometry based method for separating polyethylene glycol-6-laurate and determining the peak area of polyethylene glycol-6-laurate, the oxidation marker for the oxidative degradation of polysorbate 20, from the reference sample of step 1, and from the second sample and each optional further sample of step 2, respectively, and determining the relative peak area of polyethylene glycol-6-laurate based on the peak area of polyethylene glycol-6-laurate in the second and each optional further sample in relation to the peak area of polyethylene glycol-6-laurate in the reference sample, the relative peak area being proportional to the degree of oxidative degradation of polysorbate 20; and

step 4 optionally quantifying the amount of polyethylene glycol-6-laurate in the second sample and each optional further sample based on the relative peak area of the respective sample.

According to an example the relative peak area of polyethylene glycol-6-laurate for the second sample and each optional further sample in step 3 is determined by

- calculating the difference of the peak areas obtained, wherein the peak area of polyethylene glycol-6-laurate in the reference sample is subtracted, respectively, from the peak area of polyethylene glycol-6-laurate in the second sample and from the peak area of polyethylene glycol-6-laurate in each of the optional further sample(s);

or

- normalising the peak areas obtained, wherein the peak area of polyethylene glycol-6-laurate in the reference sample is set equal to 100% and the peak area of polyethylene glycol-6-laurate in

the second sample and in each of the optional further sample(s) is calculated respectively based thereon.

Thus, the method of the present disclosure provides the identification of polyethylene glycol-6-laurate as a byproduct of oxidative degradation of polysorbate 20 in aqueous formulations containing polysorbate 20 and makes it possible to separate, detect and determine this marker substance relative to a reference by a liquid chromatography-mass spectrometry based method (LC-MS based method) and to optionally quantify this marker substance in aqueous formulations by a specific analytical method. Since the peak area (integral) is known to be proportional to the concentration of an analyte, the relative peak area makes it possible to correlate the relative peak area of polyethylene glycol-6-laurate with the degree of oxidative degradation of polysorbate 20.

In the first alternative of the (first) method given above, the first sample of the aqueous formulation to be tested is taken as a reference sample.

In an alternative example, an externally produced or provided reference sample is used as a reference, i.e. an external reference sample is provided independently.

Therefore, according to another example it is provided a (first) method (in a second alternative) for determining the degree of oxidative degradation of polysorbate 20 in an aqueous formulation containing polysorbate 20, said method comprising the following steps:

step 1’ providing a reference sample which is an aqueous solution containing polysorbate 20, the polysorbate 20 content being the same as in the aqueous formulation to be examined;

step 2’ providing a first sample and optionally further samples of the aqueous formulation to be examined;

step 3’ using a liquid chromatography-mass spectrometry based method for separating polyethylene glycol-6-laurate and determining the peak area of polyethylene glycol-6-laurate, the oxidation marker for the oxidative degradation of polysorbate 20, from the reference sample of step 1’, and from the first sample and each optional further sample of step 2’, respectively, and
determining the relative peak area of polyethylene glycol-6-laurate based on the peak area of polyethylene glycol-6-laurate in the first and each optional further sample in relation to the peak area of polyethylene glycol-6-laurate in the reference sample, the relative peak area being proportional to the degree of oxidative degradation of polysorbate 20; and

step 4’ optionally quantifying the amount of polyethylene glycol-6-laurate in the first sample and each optional further sample based on the relative peak area of the respective sample.

According to an example the relative peak area of polyethylene glycol-6-laurate for the first and each optional further sample in step 3’ is determined by

- calculating, the difference of the peak areas obtained, wherein the peak area of polyethylene glycol-6-laurate in the reference sample is subtracted, respectively, from the peak area of polyethylene glycol-6-laurate in the first sample and from the peak area of polyethylene glycol-6-laurate in each of the optional further sample(s);

or

- normalising the peak areas obtained, wherein the peak area of polyethylene glycol-6-laurate in the reference sample is set equal to 100% and the peak area of polyethylene glycol-6-laurate in the first sample and in each of the optional further sample(s) is calculated respectively based thereon.

In an example of the disclosure, in step 2 or step 2', several samples of the aqueous formulation containing polysorbate 20 are taken at successive times T1, T2, T3.... so that not only a random sample but the course of possible oxidative degradation of polysorbate 20 in the aqueous formulation over time can be determined. For example, in step 2, two, three or more further samples of the aqueous formulation containing polysorbate 20 can be taken at successive time intervals T1, T2, T3.... which may be of the same or different lengths.

In the first method given above (first and second alternative), one aqueous formulation is tested for the oxidative degradation of polysorbate 20. However, several aqueous formulations can also be examined. Therefore, in the disclosure it is provided a (second) method for determining the degree of oxidative degradation of polysorbate 20 in a number of aqueous formulations, for example, two, three, four or more aqueous formulations, that respectively contain polysorbate 20, in particular each aqueous formulation containing the same amount of polysorbate 20, by the following steps:

step 1a providing a sample of each aqueous formulation to be examined;

step 2a using a liquid chromatography and mass spectrometry based method for separating polyethylene glycol-6-laurate and determining the peak area of polyethylene glycol-6-laurate, the oxidation marker for the oxidative degradation of polysorbate 20, from each sample of step 1a, and
comparing the peak areas of the samples with each other, wherein the difference in peak area (the relative peak area) of the samples is proportional to the degree of oxidative degradation of polysorbate 20 in each respective sample; and

step 3a optionally quantifying the amount of polyethylene glycol-6-laurate in each sample based on the obtained peak area of the respective sample.

In another aspect, the present disclosure provides a method for determining the degree of oxidative degradation of polysorbate 20 in a number of aqueous formulations each containing polysorbate 20, in particular each aqueous formulation containing the same amount of polysorbate 20, said method comprising the following steps:

step 1a providing a sample of each aqueous formulation to be examined;

step 2a using a liquid chromatography and mass spectrometry based method for separating polyethylene glycol-6-laurate and determining the peak area of polyethylene glycol-6-laurate, the oxidation marker for the oxidative degradation of polysorbate 20, from each sample of step 1a, and
comparing the peak areas of the samples with each other, wherein the difference in peak area of the samples is proportional to the degree of oxidative degradation of polysorbate 20 in each respective sample; and

step 3a optionally quantifying the amount of polyethylene glycol-6-laurate based in each sample on the obtained peak area of the respective sample.

In this (second) method of the disclosure, the peak areas can be directly compared with each other, or it is also possible to determine the relative peak area using a reference and then compare the sample(s) to the reference. The reference can be one of the samples taken, whereby the choice of the sample that can be used as a reference depends on the individual case. The reference can also be an external reference sample, as already described in connection with the first methods.

In the (second) method the relative peak area of polyethylene glycol-6-laurate in each sample may be determined in relation to a reference sample and this procedure is then carried out by

- calculating the difference of the peak areas obtained, wherein the peak area of polyethylene glycol-6-laurate in a reference sample is subtracted, respectively, from the peak area of polyethylene glycol-6-laurate in each sample of step 2a;

or

- normalising the peak areas obtained, wherein the peak area of polyethylene glycol-6-laurate in a reference sample is set equal to 100% and the peak area of polyethylene glycol-6-laurate in each sample of step 2a is calculated based thereon.

The separation of polyethylene glycol-6-laurate identified to be an oxidative marker substance of the oxidative degradation of polysorbate 20 is achieved by the liquid chromatography as part of the liquid chromatography-mass spectrometry based method by using its hydrophobic property. In the subsequent mass spectrometry procedure, the molecules to be examined are transferred into the gas phase and ionised. The molecules can be fragmented in this method. The detection and determination of the relative level of polyethylene glycol-6-laurate in step 3, step 3’ or step 2a is thus performed using the liquid chromatography-mass spectrometry based method. The optional quantification in step 4, step 4’ or step 3a may be, for example, performed with an external calibration using standard solutions of polyethylene glycol-6-laurate.

According to an example of the liquid chromatography-mass spectrometry based method the liquid chromatography is used as separation chromatography, particularly in the form of column chromatography, more particularly used as reversed-phase chromatography.

In a further example the liquid chromatography as part of the liquid chromatography-mass spectrometry based method is a high performance liquid chromatography (HPLC). Also an ultra performance liquid chromatography (UPLC) may be used.

According to an example of the liquid chromatography-mass spectrometry based method in the liquid chromatography a gradient elution is used.

In another example a polar solvent is added to the polysorbate-containing sample to be investigated before liquid chromatography is carried out, the polar solvent being in particular the solvent used in liquid chromatography.

In a further example the mass spectrometry as part of the liquid chromatography-mass spectrometry based method is selected to be a high resolution-mass spectrometry (HR-MS). High-resolution mass spectrometry may be used in particular for structure elucidation and is advantageous for this purpose.

According to an example for the data evaluation of the liquid chromatography-mass spectrometry based method of the disclosure extracted ion chromatograms are generated from a mass-to-charge ratio which comprises a range of about 487 amu, in particular comprising or consisting of a range of 486.5 to 487.5 amu.

An additional optional step 5, which can follow step 4 or step 3, or an additional optional step 5', which can follow step 4' or step 3', or an additional optional step 4a which can follow step 3a or step 2a, comprises estimating to what extent polysorbate 20 has already been oxidatively degraded in the sample based on the inversely proportional relationship between the polyethylene glycol-6-laurate level and the polysorbate 20 level. It was found that a decreasing content of polysorbate 20 in the sample and thus in the aqueous formulation clearly correlates with an increasing relative level of the oxidative marker substance polyethylene glycol-6-laurate, whereby polysorbate 20 is increasingly degraded.

According to a further example of the disclosure the aqueous formulation contains one or more proteins. The protein(s) may be selected from a monoclonal antibody, polyclonal antibody, monospecific antibody, multispecific antibody, single chain antibodies or antibody fragments thereof, such as, Fv, scFv, Fab, Fab´, scFab, F(ab')2, Fab2, Fc and Fc´-fragments, heavy and light immunoglobulin chains and their constant, variable or hypervariable region as well as Fv- and Fd-fragments, diabody, triabody, scFv-Fc, minibody, or single domain antibody or a mixture thereof. The protein(s) may be selected from therapeutic protein(s), which are used for the prevention or treatment of a disease or a disorder.

According to another example the aqueous formulation may be an aqueous pharmaceutical formulation, particularly an aqueous biopharmaceutical formulation, which contains polysorbate 20. For example, the aqueous biopharmaceutical formulation may contain pharmaceutically acceptable polysorbate 20, optionally API(s) such as one or more proteins, which are used for the prevention or treatment of a disease or a disorder, and optionally pharmaceutically acceptable excipient(s).

The polysorbate 20 present may be intended for pharmaceutical use, particularly for use in drugs. Particularly, the polysorbate 20 is pharmaceutically acceptable and may have pharma grade quality such as Ph. Eur./USP/China Pharmacopoeia grade quality. For example, the polysorbate 20 may be suitable for parenteral administration. Exemplarily mentioned are the following commercial products of polysorbate 20: Super Refined™PS20, Tween™20, Tween™20 HP, Tween™20 pharma grade or PS20 HP, particularly available from Croda, Edison, NJ, USA or Croda Europe Ltd. or PS20 China grade, particularly available from Nanjing Well Pharmaceutical Co., Ltd.

The disclosure also relates to a process (third method) of producing a recombinant protein, said process comprising the following steps:

step a) cultivating in a cell culture an eukaryotic cell expressing a recombinant protein;

step b) harvesting the recombinant protein,

step c) purifying the recombinant protein using an aqueous formulation; and

step d) using polysorbate 20, formulating the recombinant protein into a pharmaceutically acceptable aqueous formulation suitable for administration; and

step e) obtaining at least one sample of the pharmaceutically acceptable aqueous formulation of step d);

wherein the process (third method) additionally comprises performing a (first or second) method for determining the degree of oxidative degradation of polysorbate 20 as disclosed in the present disclosure with the sample obtained in step e).

According to a further example, the process (third method) for producing a recombinant protein comprises obtaining at least one sample containing the recombinant protein and polysorbate 20 in step e) as described above, wherein the sample from step d) is a drug substance sample or a drug product sample.

In another aspect, the present disclosure provides a process of producing a recombinant protein, said process comprising the following steps:

step a) cultivating in cell culture an eukaryotic cell expressing a recombinant protein in cell culture;

step b) harvesting the recombinant protein;

step c) purifying the recombinant protein using an aqueous formulation;

step d) using polysorbate 20, formulating the recombinant protein into a pharmaceutically acceptable aqueous formulation suitable for administration; and

step e) obtaining at least one sample of the pharmaceutically acceptable aqueous formulation of step d);

wherein the process additionally comprises performing a method for detecting the degree of oxidative degradation of polysorbate 20 according to any of the preceding claims 1 to 9 with the sample obtained in step e), wherein the sample from step d) may be a drug substance sample or a drug product sample.

In another aspect, the disclosure is also directed to the use of polyethylene glycol-6-laurate as oxidation marker in a method for determining the degree of the oxidative degradation of polysorbate 20 in an aqueous formulation or a number of aqueous formulations that respectively contain polysorbate 20, particularly the method as disclosed herein.

In another aspect, the present disclosure provides the use of the method as disclosed herein as a stability test during the development, manufacture, shelf life, or storage period of an aqueous formulation or a number of aqueous formulations that respectively contain polysorbate 20 for determining the degree of the oxidative degradation of polysorbate 20 in the aqueous formulation(s).

BRIEF DESCRIPTION OF THE DRAWINGS

Embodiments of the present invention will be described by way of example with reference to the accompanying figures. The figures of the present disclosure are incorporated in and constitute a part of the specification, also illustrating embodiments of the invention without limitation to the specific embodiments described. The drawings together with the general description and detailed description serve to explain the principles of the present disclosure. It is shown:

Figure 1A on the left side: extracts of two full-scan total ion chromatograms (TICs) of polysorbate 20 in an aqueous solution resulting from an embodiment of the liquid chromatography-mass spectrometry (LC-MS) based method of the invention, both shown at a mass-to-charge ratio m/z of about 227 amu (226.5 to 227.5 amu), in the upper spectrum oxidation catalyst AAPH is added, the lower spectrum is without oxidation catalyst AAPH;