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Research paper

Comprehensive physicochemical characterization of a peptide-based medicine: Teduglutide (Revestive®) structural description and stress testing

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ABSTRACT

Teduglutide (Revestive®) is a glucagon-like peptide-2 analogue used for the treatment of short bowel syndrome, a rare life-threatening condition in which the amount of functional gut is too short to enable proper absorption of nutrients and fluids. During handling prior to administration to the patient in hospital, it is possible that peptide-based medicines may be exposed to environmental stress conditions that could affect their quality. It is therefore essential to carry out stress testing studies to evaluate how such medicines respond to these stresses. For this reason, in this paper we present a strategy for a comprehensive analytical characterization of a peptide and a stress testing study in which it was subjected to various stress conditions: heating at 40 °C and 60 °C, light exposure and shaking. Several complementary analytical techniques were used throughout this study: Far UV circular dichroism, intrinsic protein fluorescence spectroscopy, dynamic light scattering, size-exclusion chromatography and intact and peptide mapping reverse-phase chromatography coupled to mass spectrometry. To the best of our knowledge, this is the first study to offer an in-depth description of the chemical structure of teduglutide peptide and its physicochemical characteristics after stress stimuli were applied to the reconstituted medicine Revestive®.

1. Introduction

Short bowel syndrome (SBS) is a serious, life-threatening condition in which the amount of functional gut is too short to enable proper absorption of nutrients and fluids. This is due to a loss of parts of the intestine or of critical intestinal function as a consequence of surgical resection, trauma or disease-association. Extensive surgical resection is the most common cause of this disease in adults. Current treatments include glucagon-like peptide-2 (GLP-2), a naturally occurring peptide, which seems to play a major role in modulating bowel adaptation and nutrient absorption. When treating patients with SBS, it is essential to maintain optimum balances of electrolytes, vitamins and nutrients, among other components. The disease can therefore be managed pharmacologically and through dietary adjustments, parenteral nutrition or intestinal transplantation [1,2].

In 2012, approval was granted to the first long-term medical treatment for SBS patients dependent on PS (parenteral support) to enhance the absorption of fluids and nutrients. Teduglutide (TGT), marketed as Gattex® in the United States and Revestive® in Europe, is a glucagon-like peptide-2 (GLP-2) analogue produced in *Escherichia coli* cells by recombinant DNA technology. It is used for the treatment of SBS patients aged 1 and over and should be administered by subcutaneous injection once daily. TGT (Fig. 1) has the same amino acid content as GLP-2, which is 33 amino acids long, the only difference being the amino acid substitution of alanine by glycine at the second amino acid residue of the *N*-terminus [3]. It is well documented that different amino acid

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residues at the *N*-terminus can lead to varying degrees of proteolysis and degradation. This means that if the *N*-terminal sequences can be modified while maintaining the required targeting specificity and affinity of the product, such modification can reduce proteolytic degradation and improve bioavailability [4]. In this particular case of TGT, this substitution results in an extended half-life compared with native GLP-2 as a consequence of a resistance to *in vivo* degradation by the enzyme dipeptidyl peptidase-IV (DPP-IV). TGT increases villus height and crypt depth [3]. Hence, TGT can reduce the PS volume requirement in patients with SBS or even assist them to become PS independent. In this way, this medicine can improve their quality of life, especially in patients with the highest PS volume requirements or with inflammatory bowel disease [5].

Biotherapeutics, especially monoclonal antibodies (mAbs), are well-established drugs used for the treatment of a wide variety of diseases. Within this field, the idea of using peptides in certain therapies is currently of increasing interest to the pharmaceutical industries. This is because peptides are smaller than other biotherapeutics (such as mAbs or Fc-fusion proteins) and they can mimic natural peptide hormones. Peptides are a unique type of biopharmaceutical compound due to their distinct biochemical and therapeutic characteristics [4,6]. Even though they are structurally less complex than mAbs and other large proteins, they are also sensitive to their environment as proteinaceous compounds.

It is therefore essential to bear in mind that peptide-based medicines could potentially be affected by environmental stress conditions in hospital settings before administration to patients, or during handling or transportation, as occur with mAbs [7,8]. Possible stress conditions include exposure to high temperatures, light or shaking [8,9]. These could cause chemical or physical degradation of the peptide, altering the quality of the biological product [10–12]. In addition, therapeutic peptides, just like other biotherapeutics, can aggregate when stored or handled in unsuitable environmental conditions. This decreases their interaction with the target. All this can have a negative effect on their therapeutic efficacy, by limiting their bioactivity or increasing their immunogenicity [13,14]. It is therefore vital to detect degraded peptides so as to ensure the efficacy and safety of the product before administration to patients [10].

Stress testing studies offer an in-depth insight into the biochemical and physical properties of biotherapeutics [15]. They provide specific information about the degradation pathways to which biotherapeutics may be exposed during handling. Normally, these studies involve exposing the medicine to relatively harsh or stressful conditions over a short period of time, e.g., high temperature, shaking, light and freeze–thaw cycles, among others [16]. These conditions are selected according to the likelihood of the biopharmaceutical product being exposed to them during the different situations in which the drug may be handled in hospital settings. Thus, these studies provide very useful information by subjecting the biotherapeutics to realistic environmental stress conditions that could arise in practical everyday situations [15].

To the best of our knowledge, neither forced degradation studies nor comprehensive physicochemical characterizations of TGT (Revestive®) have been published to date. Very recently, in the context of a wider project focused on the use/handling of this medicine in health-care facilities, we have published two works, one focused on the stability over time of the medicine in different storage conditions [17] and the other providing a quantification and identification method for TGT by (RP) UHPLC-UV-(HESI/ORBITRAP)MS [18]. Apart from our works, in the most similar previously published paper, researchers studied the formation of oligomers in TGT (no formulated in the medicine) using Sedimentation Equilibrium (SE-AUC), Sedimentation Velocity (SV-AUC), Circular Dichroism (CD) and Dynamic Light Scattering (DLS) [19]. Although this research presented valuable information, its main goal was to study the self-association of TGT monomers, and it did not therefore focus on degradation studies nor did it provide a full physicochemical characterization of TGT.

A physicochemical characterization and stress testing studies of TGT would provide essential information for an in-depth understanding of its stability, safety and efficacy [20,21]. Biotherapeutics may not always be correctly handled in the hospital pharmacy and may inadvertently be exposed to stress. It is therefore interesting to assess how these molecules behave when subjected to similar stress stimuli. In this paper we perform a comprehensive analytical characterization and a stress testing study of TGT (Revestive®) in order to expand our knowledge of this therapeutic peptide and assess its behaviour under several different stress conditions (to which it could potentially be exposed during handling in the hospital pharmacy). To make this characterisation, we used a wide variety of complementary physicochemical analytical techniques, such as Far UV CD for the assessment of the secondary structure, intrinsic protein fluorescence for the analysis of the tertiary structure, DLS to track particulate in the solutions, size-exclusion chromatography with UV detection (SE/UHPLC-DAD) to analyse oligomers and reverse-phase chromatography coupled to mass spectrometry to carry out the intact (RP/UHPLC-(Orbitrap)/MS and peptide mapping (RP/UHPLC-(Orbitrap)MS/MS) analyses in order to monitor the products of degradation (i.e. deamidation, isomerization and oxidation) by their mass.

For the stress study, four stress tests were conducted: heating at 40 °C and 60 °C (3 h), light exposure (24 h) and shaking (3 h). These tests were performed on samples of the medicine and degradation was assessed. This research presents interesting results and new insights into TGT (Revestive®), which may be very valuable for the assessment of the stability of this product during handling in hospitals. To the best of our knowledge, this is the first study to offer an in-depth description of the chemical structure of TGT peptide and its physicochemical characteristics after stress stimuli were applied to the clinical solution, i.e. Revestive® reconstituted with water for injection for parenteral use at 10 mg/mL TGT.

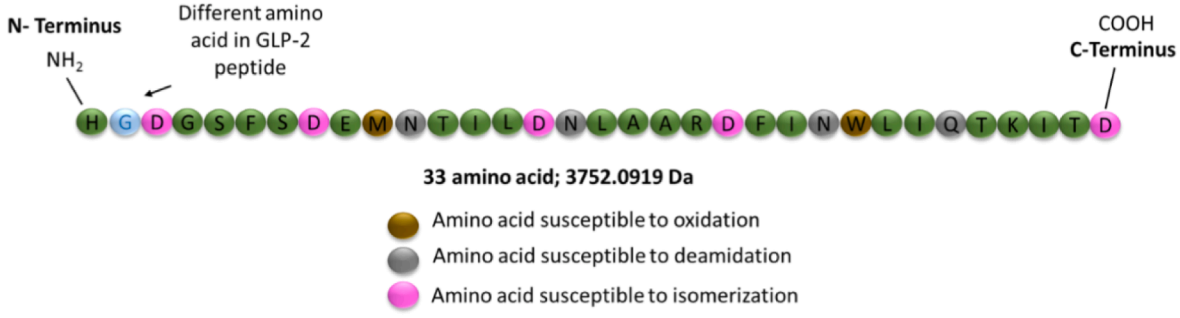

Fig. 1. Primary sequence of teduglutide.

2. Materials and methods

2.1. Materials and reagents

Reverse-osmosis-quality water was purified with a Milli-Q station from Merck Millipore (water MilliQ) (Darmstadt, Germany). Anhydrous disodium hydrogen phosphate and monohydrate monobasic sodium phosphate were supplied by Panreac (Barcelona, Spain) and Sigma-Aldrich (Misuri, USA). Water for injections solvent for parenteral use was supplied by Meinsol® (Barcelona, Spain). Acetonitrile (ACN, LC-MS purity grade) was purchased from VWR International Eurolab, S.L. (Barcelona, Spain) and formic acid (FA, LC-MS purity grade) was supplied by Thermo Fisher Scientific (Geel, Belgium). Trypsin (Trypsin Gold, Mass Spectrometry Grade) was purchased from Promega (Madrid, Spain). Ammonium bicarbonate (NH_4HCO_3), iodoacetamide (IAA), DL-dithiothreitol (DTT), acetic acid (art. A6283) and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (Madrid, Spain). Rapigest® SF surfactant was purchased from Waters (Barcelona, Spain).

2.2. Teduglutide medicine

Revestive® (Shire Pharmaceuticals Ireland Limited, Dublin, Ireland) is presented as a powder for solution and each vial contains 5 mg of TGT. After reconstitution in 0.5 mL of water for injections for parenteral use, the final concentration is 10 mg/mL. All the medicine vials used in this study were kindly supplied by the Pharmacy Unit of the University Hospital Vall d'Hebron (Barcelona, Spain).

2.3. Forced degradation study

Samples of the medicine Revestive® were subjected to forced degradation by exposure to four different conditions: (i) exposure to temperatures of 40 °C and 60 °C for 3 h in a thermomixer chamber (Eppendorf, Madrid, Spain) with one sample being subjected to a temperature ramp from 20 °C to 90 °C (for CD analysis), (ii) exposure to light irradiation (250 W/m^2) for 24 h in an aging chamber (Solarbox 3000e RH, Cofomegra, Milan, Italy), (iii) smooth shaking for 3 h at room temperature (Eppendorf Thermomixer, Madrid, Spain). When possible, all the samples (control and stressed) were analysed without dilution, i.e. in TGT medicine format (10 mg/mL). This was to make sure that the aggregation equilibrium was not altered by dilution.

2.4. Far UV circular dichroism (CD) spectroscopy

Spectra were recorded using a JASCO J-815 spectropolarimeter (Jasco, Tokyo, Japan) equipped with a Peltier system for temperature control. Temperature was set at 20 °C for all the measurements. Solution samples were studied at the target concentration of 0.2 mg/mL, diluting the medicine (Revestive®) after reconstitution (10 mg/mL) with water MilliQ. Spectra were acquired every 0.2 nm with a scan speed of 20 nm/min from 260 to 190 nm and a total of 3 measurements per sample were averaged, with a bandwidth of 1 nm. A quartz cuvette with a path length of 1 mm was used throughout the study. The blank (obtained with water MilliQ) was measured and then subtracted from the samples and means-movement smoothing was applied to all the spectra with Spectra Analysis software (Jasco, Tokyo, Japan). The sample spectrum was converted to molar ellipticity using the peptide concentration determined by UV spectroscopy (described next). Spectra were exported as ASCII files for the estimation of secondary structure content using CDSSTR and CONTINL algorithms and the SP175 and SET7 protein data sets available at the Dichroweb online server [22]. The secondary structure estimated from Far-UV CD was based on the Normalized Root Mean Squared Error Deviation (NRMSD) value; NRMSD values ≥ 0.1 for CONTINL algorithm and $0.01 \geq$ for CDSSTR algorithm were discarded as indicated in reference [23].

Also, diluted (with water MilliQ) fresh medicine samples were analysed at the concentration of 0.015 mg/mL.

2.5. UV spectroscopy

Absorbance measurements were acquired using a Hewlett Packard 8453 UV–Visible Spectrophotometer (Hewlett Packard, Germany). The UV spectra (240 – 400 nm) were registered for those TGT control and stressed samples that were further analysed by CD spectroscopy. Concentration was calculated by UV absorbance at 280 nm. For this calculation, Beer-Lambert law was used, knowing the pathlength of the quart cuvette (1 cm) and the molar extinction coefficient of TGT (5500 1/M·cm), calculated by ProtParam tool from Expasy.

2.6. Intrinsic protein fluorescence spectroscopy (IP-FS)

Measurements were carried out on a Cary Eclipse spectrofluorimeter (Agilent, Santa Clara, CA, USA). The excitation wavelength was set at 280 nm and emission was recorded from 300 to 450 nm. Reconstituted medicine (10 mg/mL) and stressed samples were analysed for fluorescence emission, exciting at 280 nm and using a 0.3 cm pathlength quartz cuvette, setting slits both for excitation and emission at 5. Fluorescence spectra were recorded at a scan speed of 600 nm/min and 3 measurements were taken, each with a total of 5 spectral accumulations. The spectral Centre of Mass (C.M.) was considered as a mathematical representation of each spectrum, and was calculated using the following equation:

$$C.M. = \frac{\sum_i^n (\lambda i f i)}{\sum_i^n f i}$$

in which λ_i is the wavelength and f_i the fluorescence intensity. This C.M was calculated from 300 nm to 450 nm.

Also, diluted (with water MilliQ) fresh reconstituted medicine samples were analysed at the concentration of of 0.015 mg/mL at room temperature in a 45 μL fluorescence quartz cuvette.

2.7. Dynamic light scattering (DLS)

DLS readings were carried out on a Zetasizer Nano-ZS90 (Malvern, UK). A 50 μL volume thermostatted high precision cell with a path length of 3 mm was used throughout (Hellman Analytics, Germany). Automatic readings were selected with an acquisition time of 10 s each, at a fixed temperature of 20 °C. The recorded data were analysed using Zetasizer Sofware ver. 7.13 (Malvern Panalytical, Malvern; UK). Size distribution was estimated using CUMULANT method. Protein analysis model was used to process the size distribution results. Measurements were obtained for the reconstituted medicine (TGT 10 mg/mL) and the stressed samples of the reconstituted medicine. A diluted sample (with water MilliQ) at 1 mg/mL TGT was also analysed.

2.8. Intact reverse phase chromatography (RP/UHPLC-(Orbitrap)/MS)

The chromatographic method used here was previously developed and validated by the authors [18].

A proper analytical platform was used to perform the method (Thermo Scientific, Waltham, MA, USA) equipped with two ternary pumps, a degasser, an autosampler, and a thermostatted column compartment. The chromatograph was coupled in line to a Q-Exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Scientific, Waltham, MA, USA). The ionization was performed using a heated electro spray ionization (HESI) source. The chromatographic instrument was managed by Xcalibur® 4.0 software (Thermo Scientific, Waltham, MA, USA) and the mass spectrometer by Tune® Software (Thermo Scientific, Waltham, MA, USA).

Chromatographic separation was conducted using an Acclaim Vanquish C18, 2.2 mm, 2.1 mm × 250 mm column (Thermo Fisher

Scientific, Waltham, MA, USA). The flow rate was 0.3 mL/min and 5 μL of samples were injected into the column. The column temperature was 25 °C. The eluent system was composed of 0.1 % FA in deionized water (mobile phase A) and 0.1 % FA in ACN (mobile phase B). The column was equilibrated with 30 % of eluent B for 5 min. Then a linear gradient was applied from 30 % to 90 % of eluent B for 5 min, and kept constant for 2 min. In order to recondition the column, the gradient was reduced to 30 % of eluent B for 1 min. Total analysis run time was therefore 13 min. All TGT samples were analysed at 15 mg/L, the target concentration optimized in [18].

The MS instrument was operated in positive mode ($M-H^+$) at a mass range of 300 *m/z* to 4000 *m/z* using 17,500 resolution. The subsequent MS settings were as follows: spray voltage 3.8 kV, sheath gas flow rate 40 AU, auxiliary gas flow rate 10 AU, capillary temperature 320 °C, AGC target value of 3.0×10^6, S-Lens RF Level 50, max injection time 100 ms and number of microscans 1.

2.9. Size exclusion chromatography (SE/HPLC-DAD)

The analysis was performed by liquid chromatography using an Agilent 1100 chromatograph equipped with a quaternary pump, degasser, autosampler, column oven and photodiode array detector (Agilent Technologies, USA). Drug chromatographic evaluation was carried out in a *SEC*-5 column, 5 μm 150 Å, 4.6 × 300 mm (Agilent Technologies, USA) in which temperature was set at 25 °C. The flow rate was 0.35 mL/min and the mobile phase was composed of 150 mM of phosphate buffer pH 7.0, which was prepared with anhydrous disodium hydrogen phosphate and monohydrate monobasic sodium phosphate. An isocratic elution mode was applied for 20 min. 1 μL of the reconstituted medicine (TGT 10 mg/mL) and the stressed samples (of the reconstituted medicine) were injected into the column. The UV–visible spectra were recorded between 190 and 400 nm with a data point every 0.5 nm. Chromatograms were registered at 214 nm with a reference band at 390 nm.

2.10. Tryptic digestion of the samples

The tryptic digestion was performed in 0.5 mL Eppendorf tubes. 50 μg of TGT were diluted to 2 mg/mL in buffer digestion (NH_4HCO_3, 5 0 Mm, at pH 7.8). The peptide was denaturalized with the addition of 50 μL of Rapigest® surfactant (0.1 % reconstituted in buffer digestion) and was incubated at 80 °C for 20 min with a post reaction cooling down to room temperature. Then, the disulphide bond reduction was performed with DTT 20 mM for 60 min at 37 °C and subsequent alkylation with IAA 60 mM at 25 °C in darkness for 30 min. After the alkylation step, 6 μL of trypsin (0.5 μg/μL, 1:33 protein ratio) were added to the solution and incubated for 16 h at 37 °C. The reaction was quenched with 10 μL of TFA (25 % in water) and incubated for 30 min at 37 °C. The resulting cloudy (Rapigest® precipitates) solution was centrifuged at 13,000 rpm for 10 min and the supernatant was transferred to an insert which was kept in amber vials. 5 μL of this solution were used to separate and monitor the tryptic peptides by LC-MS/MS (the chromatographic method is described below in Section 2.11 "*Peptide mapping (RP/UHPLC-(Orbitrap)MS/MS) analysis*").

2.11. Peptide mapping (RP/UHPLC-(Orbitrap)MS/MS) analysis

The peptide mapping analysis was performed using the analytical platform described in Section 2.8. "*Reverse phase chromatography (UHPLC (HESI/ORBITRAP)MS)*". The tryptic peptides were separated and monitored by LC-MS/MS analysis using an Acclaim Vanquish C18 Column, 2.2 mm, 2.1 mm × 250 mm (Thermo Scientific).

Analysis was performed using a binary gradient of 0.1 % (v/v) formic acid in water (mobile phase A) and 0.1 % (v/v) FA in ACN (mobile phase B). Gradient conditions were as follows: 2 % B increased to 40 % B in 45 min with a further increase to 80 % B in 1 min. The gradient was kept at 80 % B for 4 min, and then shifted to 2 % B in 0.5 min. Lastly, 2 % B was maintained for 5 min for column reconditioning. The column temperature was kept at 25 °C throughout the study and the flow rate was set at 300 μL/min. The subsequent HESI settings were as follows: spray voltage 3.8 kV, sheath gas flow rate 40 AU, auxiliary gas flow rate 10 AU and capillary temperature 320 °C. The MS method consisted of full positive polarity MS scans at a resolution setting of 70,000 (at *m/z* 200) with the mass range set to 200–2000 *m/z* and an AGC target value of 3.0×10^6, a maximum injection time of 100 ms and 1 microscan. In-source CID was set to 0 eV. MS^2 settings were as follows: a resolution setting of 17,500 (at *m/z* 200), an AGC target value of 1.0×10^5, the isolation window was 2.0 *m/z* and maximum IT was 200 msec.

2.12. Data processing

Intact peptide quantification was performed using Xcalibur® QualBrowser 4.0 for signal integration. The peptide mapping data processing, quantitation and identification were performed on BioPharma Finder 3.1 software (Thermo Scientific) using the parameters summarized in Supplementary Material Table S1. The secondary structures were estimated using CDSSTR [24] and CONTINL [25] algorithms and the SP175 protein DataSet [26] available at the Dichroweb website [27].

3. Results

3.1. Visual inspection

A quick visual inspection revealed that the samples remained clear after exposure to the stress conditions. No precipitates or particulate matter were detected with the naked eye. No colour changes or turbidity were observed at any time during the study except in the samples submitted to accelerated light exposure, in which a yellow color was observed (Supplementary material, Imagen S1) at the end of the test that was kept stable along time with no turbidity.

3.2. Far UV circular dichroism (CD) spectroscopy

It is well-known that the secondary structures of peptides and proteins can be estimated via CD [28,29]. Changes in the secondary structures of peptides or proteins could be related with a possible decline in their therapeutic activity [21,30].

The fresh and stressed samples of TGT were diluted in water to 0.2 mg/mL and then characterized by Far UV CD. This dilution was previously optimized using fresh TGT samples to minimize the effect of the excipients (salts from buffers mainly) on the detector which prevent from recording the CD spectra. The characteristic Far UV spectra for fresh TGT and stressed samples are shown in Fig. 2. The fresh TGT UV CD spectra are characterized by the presence of two negative maxima at 201.2 ± 0.2 nm and 228.2 ± 0.6 nm and a positive maximum at 216.0 ± 0.6 nm. This CD spectra slightly differs from the spectra of TGT drug substance in reference 19 where is also demonstrated the dependence of the TGT CD spectra with the concentration, mainly due to TGT self-association. The CD spectra in reference 19 at 0.1 mg/mL share the negative maximum at 201 nm, being different from about 210 nm with a shift of the second negative maximum to 222 nm (instead of 227 nm, in the present work). These CD spectral differences can be attributed that here, TGT is studied as it is formulated in the marketed medicine, therefore including the excipients [3] in the media.

The above TGT spectral checkpoints were tracked in the stressed samples so as to detect changes in the shape of the CD spectra (Table 1). As can be seen in the spectra (Fig. 2) and in the control checkpoints, the results for the temperature (40 °C and 60 °C) and the shake stress tests were similar to those obtained for the fresh TGT sample. By contrast, the CD spectrum for the light stress sample was different from that of the control sample, indicating changes in the secondary structure of TGT (Fig. 2, and Table 1).

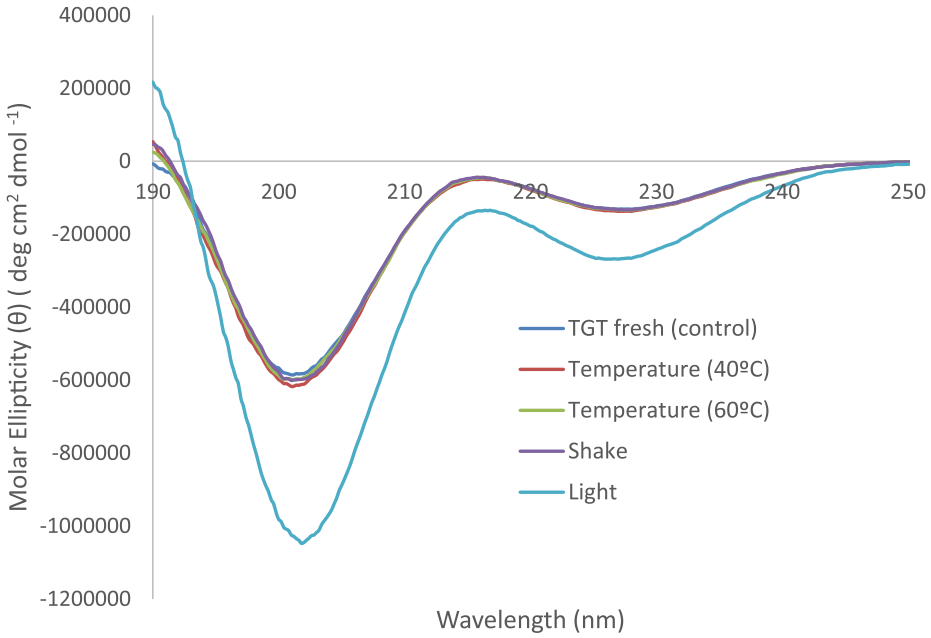

Fig. 2. Representative Circular Dichroism spectra for a control sample of TGT (Revestive®) compared with stressed samples. Spectra recorded at 0.2 mg/mL TGT diluted with water MilliQ from Revestive® (reconstituted with water for injection for parenteral use at 10 mg/mL TGT).

Table 1
Performance characteristics of UV CD spectra of TGT (Revestive® reconstituted with water for injection for parenteral use at 10 mg/mL) control and stressed samples, recorded diluted with water MilliQ at 0.2 mg/mL.

TGT Sample	Negative Maximum 1 (nm)	Positive Maximum (nm)	Negative Maximum 2 (nm)
Control	201.0 ± 0.2	216.0 ± 0.4	228.2 ± 0.6
Temperature (60 °C), 3 h	201.2	216.0	227.8
Temperature (40 °C), 3 h	201.2	215.2	227.8
Light stress, 24 h	201.8	216.4	226.4
Smooth shaking, 3 h	201.2	216.2	227.8

The percentage of secondary structures for the fresh and stressed samples of TGT (Revestive®) was therefore estimated. Two of the most suitable protein data sets were checked, i.e. SP175 and SET7, and applying two algorithms, i.e. CDSSTR and CONTINL. From the NRMSD values, which indicates the "goodness-of-fit" [23], the best results were obtained by using the SET7 protein data set and the CDSSTR algorithm (NRMSD = 0.01). Table 2 shows these results. The estimated secondary structure of the fresh control TGT sample is characterized by the presence of a majority content in random coil (45 %) and β-sheet (27 %) conformation, also an 8 % of α-helix is proposed, being similarly as in reference 17 (38 % random coil, 23 % β-sheet and 12 % α-helix. As expected, the observed changes (Fig. 2) in the CD spectrum after accelerated light exposure were reflected in the estimation of the percentage of the secondary structure types (Table 2), highlighting a decrease from 45 % to 24 % in the random coil and an increase from 8 % to 43 % in the α-helix; also turns and β-sheet percentage showed decrease in their percentage with respect to the control unstressed TGT sample. The percentage of secondary structure in the samples subjected to temperatures of 40 °C and 60 °C and shake stresses was the similar as in the fresh control TGT samples (Table 2).

Table 2
Percentage of estimated secondary structures for representative fresh and stressed samples of TGT (Revestive®). For the CD analysis, the samples were diluted with water MilliQ from 10 mg/mL (reconstituted medicine) to 0.2 mg/mL or 0.015 mg/mL.

	Stress	α-helix (%)	β-sheet (%)	Turns (%)	Random coil (%)
0.2 mg/mL	Control	8	27	20	45
	Temperature (60 °C) 3 h	7	30	18	44
	Temperature (40 °C) 3 h	8	29	20	44
	Light stress 24 h	43	21	12	24
	Smooth shaking, 3 h	7	31	20	40
0.015 mg/mL	Control	7	25	18	50
	Light stress 24 h	48	21	9	22

In addition, the thermal stability was checked by applying a temperature ramp from 20 °C to 90 °C to a fresh TGT sample (diluted from the reconstituted medicine to 0.2 mg/mL). As expected, the characteristic shape of the UV CD spectra changed gradually as temperature increased (Supplementary Material Fig. S1). Based on the observation of these changes, we could infer that the gradual decrease in the ellipticity could be related with a progressive decrease of the unordered structures (negative maximum at 201 nm) and with a slight increase in the α-helix content (positive band at around 193 nm). An abrupt change was observed between 85 °C and 90 °C, nevertheless peptide denaturalization was not achieved at this high temperature of 90 °C since the CD spectrum remains with characteristic bands (not a flat spectrum).

As last, a CD spectrum at the TGT concentration of 0.015 mg/mL was compared with the CD spectrum of the 0.2 mg/mL (Table 2 and Supplementary Material Fig. S2) in order to estimate whereas this smaller concentration -further used to the intact TGT analysis- affects the secondary structure content. It seems that there is a slight modification between the two dilutions, slightly increasing the random coil structure with the dilution (estimated from 45 % to 50 %).

3.3. *Intrinsic protein fluorescence spectroscopy (IP-FS)*

In principle, Intrinsic Tryptophan-FS (IT-FS) of proteins can provide a wealth of information regarding the location of the tryptophan within their macromolecular structure, which in turn offers information about

the conformation of the tertiary structure. Changes in fluorescence intensity are strongly dependent on changes in the close surroundings of the tryptophan residues, i.e. folding/unfolding, structural rearrangements, etc. An excitation wavelength of 298 nm is commonly used in IT-FS to avoid the interference of the tyrosine which is not excited at this wavelength. Nevertheless, TGT has only one tryptophan residue and without tyrosine in its sequence. Therefore, we have used IP-FS instead of IT-FS to study TGT tertiary conformation.

Fig. 3 shows the fluorescence emission spectra and Table 3 gathers the calculated C.M. These results suggested that there were not conformational modifications promoted by the 40 °C and 60 °C (for 3 h) and by shaking the samples since similar emission spectra and therefore, similar calculated C.M were observed. Sample submitted to accelerated light exposition significantly modified its conformation, this indicated by the substantial spectrum and C.M. changes. In addition to a great intensity decrease, a red spectral shift in this sample was observed, which will be further addressed in the Discussion Section.

As in the CD studies, an emission fluorescence spectrum at the TGT concentration of 0.015 mg/mL was registered and compared with that of the TGT in the reconstituted medicine (10 mg/mL) in order to estimate whereas this smaller concentration -further used to the intact TGT analysis- affects the tertiary conformation. Slight modifications were detected in the calculated C.M.: 358.6 nm for TGT 10 mg/mL and 363.4 nm for TGT 0.015 mg/mL. These results suggested that the conformation was slightly modified at this lower concentration, which is addressed in the Discussion Section.

3.4. Dynamic light scattering (DLS)

Representative results for DLS analyses are shown in Fig. 4 (volume size distribution graphs) and Table 4 (Hydrodynamic diameter (D_h), abundance population and estimated molecular weight of the populations). In the control sample, TGT peptide is characterized by a single population of particles with an mean D_h of 4.454 nm, whose molecular weight was estimated in 21.9 ± 3.8 kDa according to a globular protein standard; this size corresponds to hexamers of TGT (3.752 kDa), and taken into account the SD values (0.737 for D_h and 3.8 for MW) self-assemblies of pentamers and heptamers could be also present at lower proportions in the 10 mg/mL reconstituted solution of the medicine. For the 40 °C heat stress, no modifications in the size distribution were observed; however, for the smooth shaking and 60 °C heat stresses slight changes were detected, being these similar. In both cases the D_h increased to 4.66 nm, corresponding to an estimated molecular weight

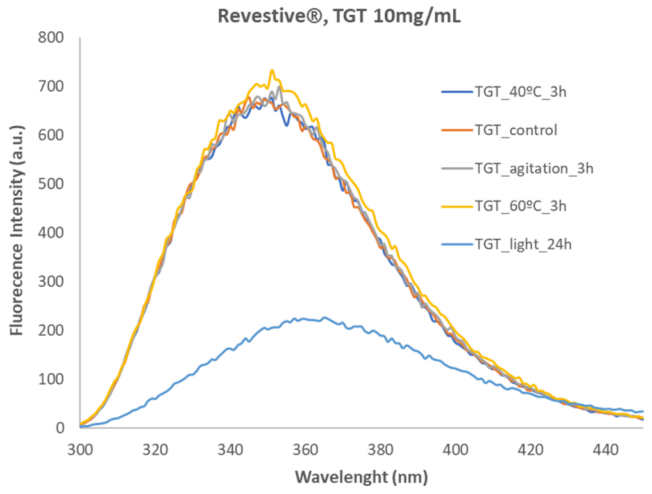

Fig. 3. Representative fluorescence emission spectra of control and stressed samples of TGT (Revestive® reconstituted with water for injection for parenteral use at 10 mg/mL TGT).

Table 3
TGT (Revestive® reconstituted with water for injection for parenteral use at 10 mg/mL) tertiary structure assessed by means of the centre of spectral mass (C.M.) of the fluorescence spectrum.

Stress	Centre of spectral mass (C.M.), nm (λ exc 280 nm, IP-FS)
Control	358.6
Temperature (60 °C), 3 h	359.2
Temperature (40 °C), 3 h	358.6
Light stress, 24 h	371.8
Smooth shaking, 3 h	358.8

of 24.4 kDa, therefore suggesting hexamers/heptamers, which is not unusual, since with the increase of the temperature, the aggregation of the proteinaceous material is expected, and also by agitation. However, the results of the light accelerated exposure sample showed significant changes in the size distribution with respect to the unstressed control TGT sample. The population peak detected in the control samples was split into two new populations: the main (64 %) with a D_h of 2.228 nm, and the other (36 %) with a D_h of 5.118 nm, with the associated molecular weight of 4.3 kDa (close to the TGT molecular weight of 3.752 kDa) and 30.3 kDa (octamers) respectively. For this last peak population with the greater D_h, the SD associated to the estimated molecular weight (7.7) it could be inferred the presence in the sample TGT association from 5 to 10 molecules.

As the TGT self-association is concentration dependant [19], a diluted (with water MilliQ) sample of 1 mg/mL was analysed and compared with a sample of reconstituted medicine (TGT 10 mg/mL). The results showed in Fig. 4B and in Table 4 indicate the shift of the single peak population detected in the size distribution graphs from hexamers/heptamers at TGT 10 mg/mL to monomers at the concentration of 1 mg/mL.

3.5. Intact reverse phase chromatography (RP/UHPLC-(Orbitrap)/MS analysis

Intact mass analysis was performed on fresh (control) and stressed samples of TGT under a denaturing condition by RP/UHPLC-(Orbitrap)/MS analysis. The aim was to characterize the chemical structure of TGT and to detect possible degradation products produced by the various stress tests.

Results are showed in Fig. 5 (overlaid chromatograms in Supplementary material Fig. S3). Fresh TGT was characterized by a single mass of 3751.7934 Da. This corresponds to the average theoretical mass of TGT with no post-translational modification (PTMs), as indicated in the European Public Assessment Report on Revestive® [3].

When the samples were subjected to the temperature (40 °C and 60 °C) and shake stresses, no chemical modifications were observed in the intact mass spectrum as compared to the fresh sample. This suggests that the chemical structure of TGT has not undergone significant changes. The chromatograms were characterized by a single peak with a deconvoluted mass of 3752,0919 Da, therefore corresponding with the TGT molecule with no self-associations, taken into account that all the samples for intact anlysis were diluted at the target concentration of 15 mg/L. Nevertheless, the light stress induced the partial degradation of TGT. The intact mass of TGT (3751.8147 Da) and several intact mases were detected to correspond with products of TGT oxidation i.e. 3767.8071 Da (+1 oxidation); 3783.8040 Da (+2 oxidations); 3799.7946 Da (+3 oxidations).

3.6. Size exclusion chromatography (SE/HPLC-DAD) analysis

One of the first signs of proteinaceous drugs degradation is the formation of aggregates. Fresh and stressed samples of TGT were analyzed by SE-HPLC/DAD in order to obtain their *SEC* aggregates profile and estimate the presence of aggregates/fragments if occurred. As in the

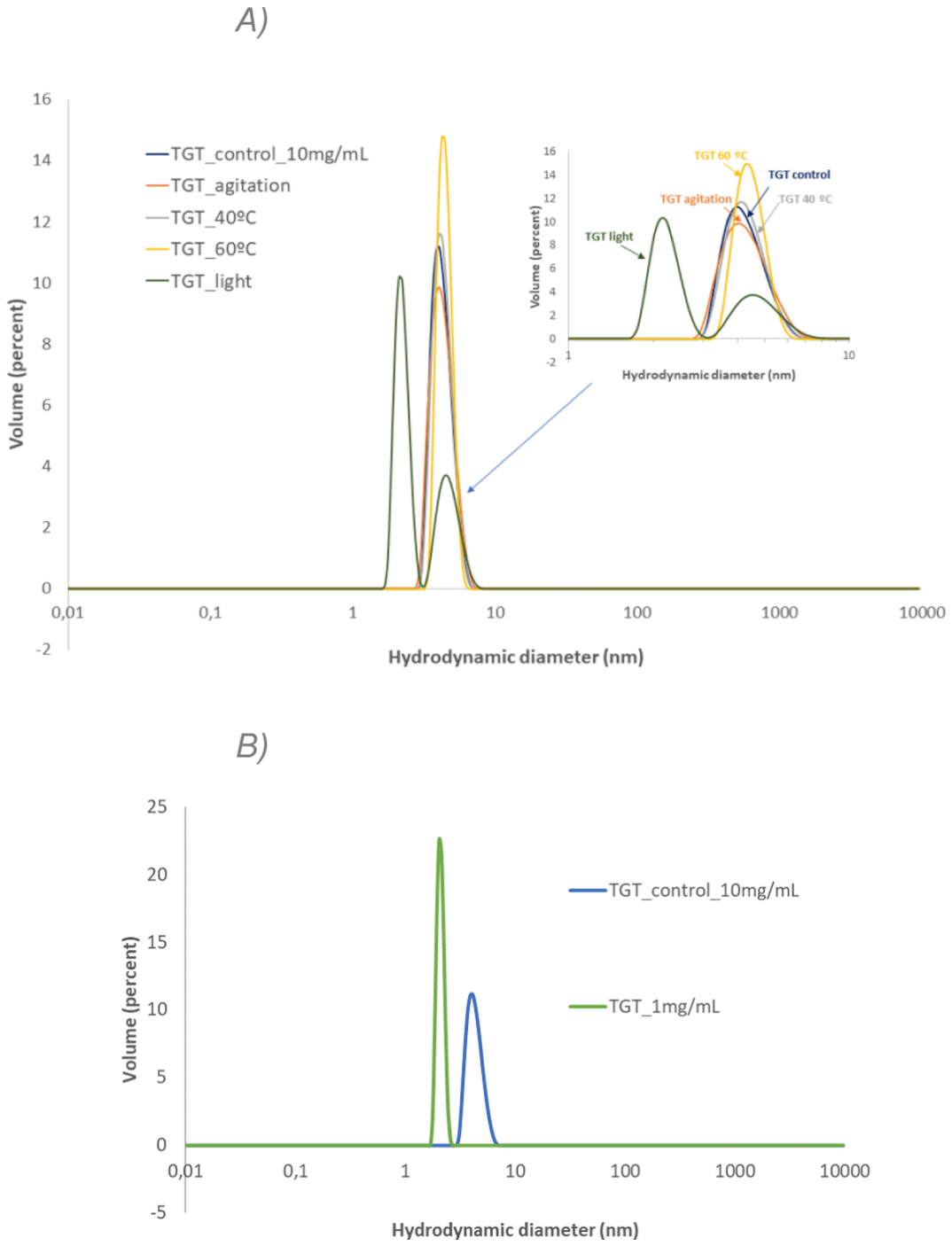

Fig. 4. A: Representative size distribution graphs by volume of control and stressed samples of TGT (Revestive® reconstituted with water for injection for parenteral use at 10 mg/mL TGT), a zoom of D_h from 1 nm to 10 nm is shown in the window. B: TGT control samples at 10 mg/mL (Revestive® reconstituted with water for injection for parenteral use) and at 1 mg/mL (dilution with water MilliQ of the reconstituted medicine).

above studies, the TGT reconstituted medicine was analyzed immediately after opening the vial (fresh/control sample at 10 mg/mL TGT) and the resulting profile was used as a control for comparing with the chromatograms for stressed TGT samples in order to detect any changes. Samples were analysed without further dilutions. Representative SE chromatograms of fresh TGT (control) and TGT under stress condition are shown in Fig. 6 (overlaid chromatograms in Supplementary material Fig. S4). The retention times and percentage of the peaks detected in the chromatograms are indicated in Table 5. The percentages of the peaks were estimated considering as 100 % the sum of the areas under all the peaks in the chromatogram, and calculating the corresponding percentage for each individual peak.

The control *SEC* profile in TGT is characterized by a single peak which elutes at 11.36 ± 0.01 min. This peak could be attributed to a single population of TGT with similar hydrodynamic volume. No high or low molecular weight species (HMWS or LMWS) were detected in this control sample.

The results of TGT samples submitted to temperature stress (40 °C and 60 °C for 3 h) suggested an incipient and gradual TGT aggregation process as temperature increased (Fig. 6 green and pink lines, Table 5). A small peak (10.56 ± 0.04 min) which appears prior to the main peak can be attributed to the presence of TGT HMWS (estimated about 6 % and

Table 4
Population average D_h of TGT (Revestive®) control and stressed samples and their respective estimated molecular weight.

	Hydrodynamic Diameter, D_h (Mode ± SD)	Abundance (%)	Molecular Weight, MW (Mode ± SD)
Control (10 mg/mL)[1]	(4.454 ± 0.737) nm	100	(21.9 ± 3.8) kDa
40 °C (10 mg/mL)[1]	(4.454 ± 0.695) nm	100	(21.9 ± 3.6) kDa
60 °C (10 mg/mL)[1]	(4.666 ± 0.526) nm	100	(24.4 ± 2.7) kDa
Agitation (10 mg/mL)[1]	(4.666 ± 0.832) nm	100	(24.4 ± 2.7) kDa
Light stress (10 mg/mL)[1]	(2.228 ± 0.244) nm	64	(4.3 ± 0.8) kDa
	(5.118 ± 0.888) nm	36	(30.3 ± 7.7) kDa
Control (1 mg/mL)[2]	(2.126 ± 0.145) nm	100	(3.9 ± 0.3) kDa

(1) Revestive® reconstituted with water for injection for parenteral use at 10 mg/mL.
(2) Reconstituted Revestive® at 10 mg/mL diluted with water MilliQ at 1 mg/mL.

14 % of the total areas under 40 °C and 60 °C temperature stress respectively). Similar aggregation behavior was induced by the shake stress test (Fig. 6 red line, Table 5) and an HMWS peak was found at 10.56 ± 0.04 min (estimated as 5 % of chromatographic area). The degradation promoted by light exposure (24 h) led to the transformation of the main TGT species into HMWS (6.79 ± 0.04 min, estimated as 21 % of the total area) in 24 h (Fig. 6 olive green line, Table 5). These aggregates or HMWS promoted by the light stress had a higher range of molecular weights than the aggregates found in case of the temperature or shake stress tests (wider peak at shorter retention times). The results also suggests that accelerated light exposition could promote the formation of LMWS with respect the main TGT species as a small peak was detected at 12.91 ± 0.03 min, which corresponds to 5 % of the total estimated area).

3.7. Peptide mapping (RP/UHPLC-(Orbitrap)MS/MS) analysis

Peptide mapping is the gold standard analytical method used to assess the critical quality attributes (CQAs) of biotherapeutic products related with the chemical structure. It is used to confirm the identity of a drug substance by providing information on its amino acid sequence (primary structure). It also provides site-specific information about post-translational modifications (PTMs), such as degradative events (oxidation, deamidation and isomerization), which require close monitoring [31]. Tryptic digestion of reconstituted TGT (10 mg/mL) fresh and stressed samples was done in order to identify and quantify some CQAs (deamidation, isomerization and oxidation). Fig. 7 shows the chromatograms obtained from peptide separation of fresh and stressed samples of reconstituted TGT. To ensure the reliability of the results, digestions were performed in triplicate. The efficiency of the peptide digestion was studied with the percentage of sequence coverage. One hundred percent sequence coverage was attained for all of the samples under investigation (data not shown).

Post-translational modifications are the main focus when evaluating the integrity of a biopharmaceutical molecule. As mentioned earlier, changes in attributes such as oxidation, deamidation or isomerization can be detrimental to the efficacy, stability and immunogenicity of therapeutic proteins and must therefore be closely monitored. Some of these modifications may also be induced by the digestion process itself or could be already present in the original sample (the reconstituted medicine). Then, fresh reconstituted TGT sample was also digested and analysed in order to identify and quantify these modifications, which enabled us to distinguish modifications already present in the fresh sample from those produced by the physical stresses. Relative levels of each PTM were studied and quantified using triplicate sample analysis, producing good standard deviation, even in species with low abundance. After digestion with trypsin, three peptide fragments (i.e. HGDGSFSDEMNTILDNLAAR, DFINWLIQTK and ITD) were detected with associated PTMs. A total of 8 PTMs were reported in fresh and stressed samples of TGT, with 4 sites identified as containing deamidations, 2 sites containing oxidations and 2 sites containing isomerizations. Fig. 8 shows the average relative abundance of the reported PTMs in control and stressed samples of TGT.

Deamidation is the most likely modification to affect therapeutic

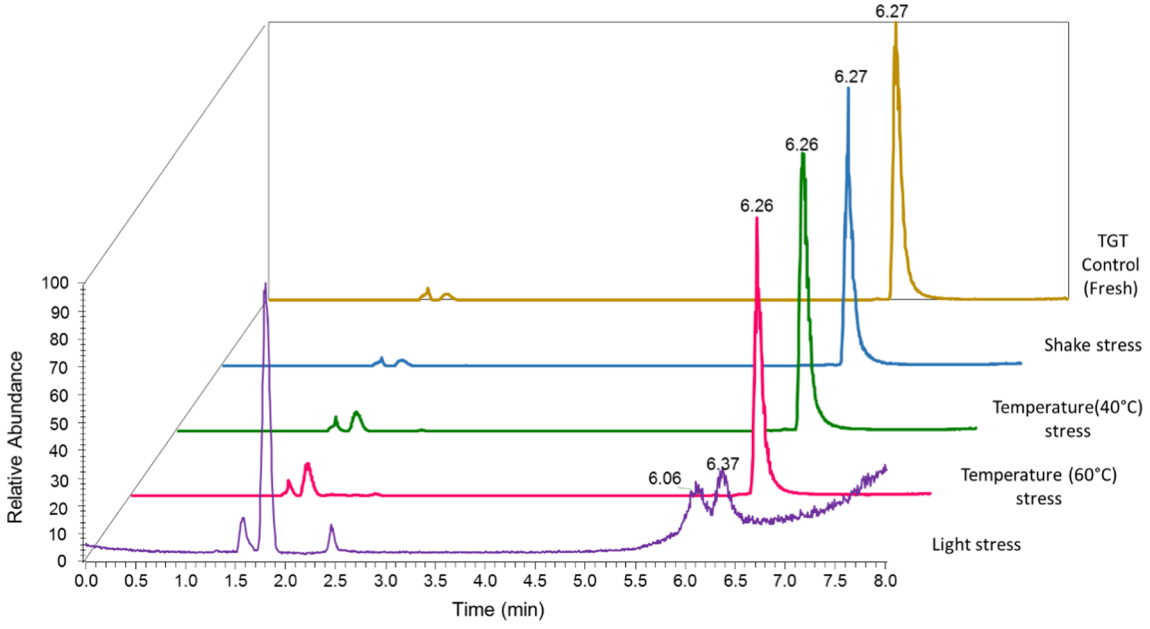

Fig. 5. Representative intact RP/UHPLC-(Orbitrap)/MS chromatograms for a control sample of TGT (Revestive®) compared with stressed samples. Chromatograms recorded at 0.015 mg/mL TGT diluted with water MilliQ from Revestive® (reconstituted with water for injection for parenteral use at 10 mg/mL TGT).

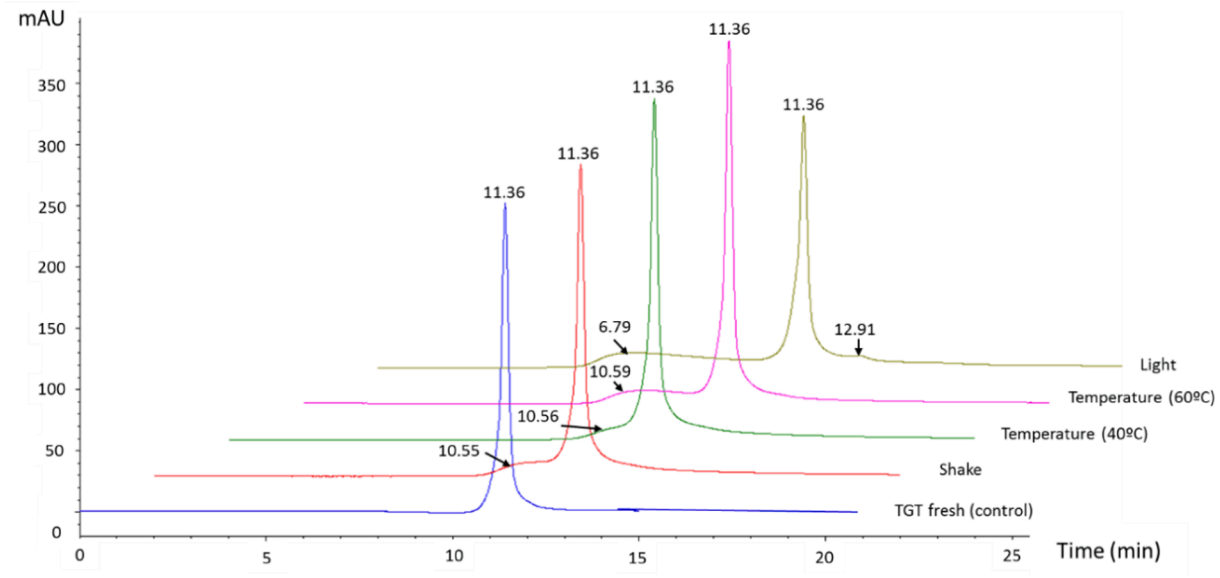

Fig. 6. Representative SE/HPLC-DAD chromatograms for control and stressed samples of TGT (Revestive® reconstituted with water for injection for parenteral use at 10 mg/mL TGT).

Table 5
Overall results for experimental retention time and abundance in *SEC* analysis: TGT (Revestive® reconstituted with water for injection for parenteral use at 10 mg/mL).

Stress	peak	Retention time (min)	Abundance (%)
Control	1	11.36	100
Temperature (60 °C), 3 h	1	10.55	14
	2	11.36	84
Temperature (40 °C), 3 h	1	10.56	6
	2	11.36	94
Light stress, 24 h	1	6.79	21
	2	11.36	74
	3	12.91	5
Smooth shaking 3 h	1	10.55	5
	2	11.36	95

proteins that have been stored for long periods. Glutamine (Q) and asparagine (N) residues are prone to suffer deamidations, and deamidations of both these residues were found in both fresh and stressed samples of TGT (Fig. 8A). As mentioned earlier, 4 amino acids were deamidated in the N11, N16, N24 and Q28 positions. The relative abundances of deamidation were very low (<2.5 % in all cases) and were unaffected by the stress conditions applied. Deamidations have been reported to be commonly triggered by the aggressive chemical conditions resulting from tryptic digestion [45]. This suggests that the low abundances found in control and stressed samples of TGT could be due to the digestion process effect, but it also could be attributed to a minimal percentage of this chemical modification in the fresh TGT sample. On the other hand, the rate of deamidation varies greatly from one site to another, with some positions more prone to deamidation than others. The results indicate that the stress conditions did not affect the deamidation rate of either N or Q in TGT at the conditions as it is formulated in the reconstituted medicine Revestive®.

Another possible consequence of long-term storage of therapeutic proteins is aspartate (D) isomerization, which has been described as a pH-dependent reaction [33]. D isomerization is a critical CQA which gives rise to immunogenicity [34], and must therefore be closely monitored to ensure the quality, efficacy and safety of the drug. Like deamidations, isomerizations are treatment-induced PTMs. The position of aspartate in the primary sequence will determine the likelihood of the peptide undergoing isomerization. Regarding this, D3 and D21 suffered isomerization in fresh TGT, albeit in almost unappreciable percentages (<1 %) (Fig. 8B). D isomerization levels in the different stressed samples were very similar to those in the control samples, indicating that this PTM was not induced by any of the conditions tested and was a result of the digestion protocol or it was already present at this.

In biotherapeutic drugs, oxidation usually takes place in cases of excessive handling or exposure to light [35]. It potentially induces protein aggregation, increasing the immunogenicity of the drug [35] and impairing its biological activity. Typically, sulphur-containing residues (methionine, cysteine) and aromatic residues (phenylalanine, tryptophan, tyrosine) are the most prone to suffer oxidation reactions [36]. TGT contains 2 oxidation sites i.e. methionine (M10) and tryptophan (W25) (Fig. 8C). In fresh TGT, low levels of oxidation (<1 %) induced by the digestion process were found in both amino acids prone to suffer oxidation (M10 and W25). In the stressed samples, a clear increase in oxidation levels was observed in the samples subjected to light stress, especially for M10 residue, which reached an 80 % abundance level. Light exposure also affected W25, in which an increase in oxidation from roughly 0.3 % (fresh TGT) to 12 % was observed. Another interesting finding is that methionine is more prone to undergo oxidation than tryptophan in TGT. M10 and W25 are involved in the process by which TGT binds to its target (GLP-2R) [37]. This means that oxidations in these positions can hinder the biological activity of TGT and increase its immunogenicity, thus impairing the safety and efficacy of the drug. Furthermore, high levels of oxidation are related to the formation of aggregates, which is undesirable [37]. Lastly, a slight increase (roughly 1.0 % to 1.4 %) in M10 oxidation was found in the samples subjected to 60 °C temperature stress. This seems unlikely to have any significant effects on the quality of the drug as the percentage of oxidation is almost negligible. The shake and 40 °C temperature stress tests did not affect TGT oxidation in any significant way.

4. Discussion

TGT is a recombinant human GLP-2 analogue-based drug synthetized by a genetically modified strain of *E.coli*. Structurally, it is made up of a single chain of 33 amino acids, and it has no disulphide bonds or glycosylation sites [38].

Different analytical strategies and techniques were used for a comprehensive study of reconstituted TGT (Revestive®,10 mg/mL) medicine, in order to further compare the results with samples

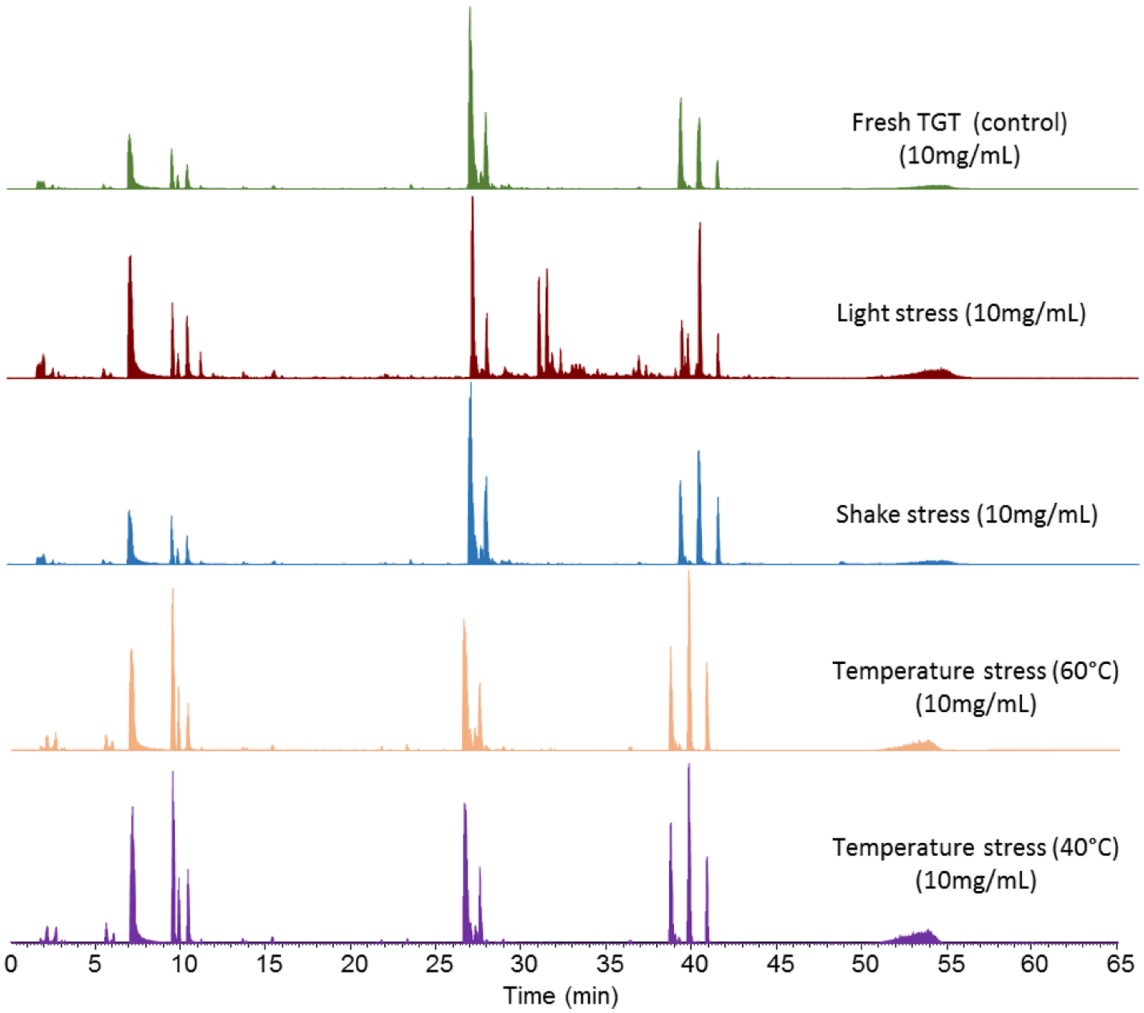

Fig. 7. Total Ion Chromatograms (0–65 min) obtained from peptide mapping experiments on control and stressed samples of TGT (Revestive® reconstituted with water for injection for parenteral use at 10 mg/mL TGT).

submitted to different environmental stresses. Although the target concentration was TGT 10 mg/mL -the concentration administered to patients-, diluted samples were analysed due to the limitations of some analytical techniques, such as CD (0.2 mg/mL) and LC/MS (0.015 mg/mL). In previous research conducted from freshly thawed aliquots of TGT drug substance (14.0 mg/mL in 17.5 mM sodium phosphate, pH 7.4), the TGT self-association (natural aggregation) dependence on concentration was demonstrated [19]. Also previously, the tendency of GLP-2 and related peptides to self-associate was proposed [43]. Only an NMR study indicated GLP-2 to be monomeric at a relative high concentration (7.5 mg/mL) but salts were absent in the medium which contains trifluoroethanol [44]. Results from the present work are consistent with this finding, taken into account that here TGT has been studied in its commercial formulation, therefore, in presence of excipients [3]. Then, DLS and *SEC* (10 mg/mL TGT reconstituted medicine) analyses confirm the presence of a single size (hydrodynamic volume) population. DLS protein analysis tool suggests a dynamic mixture of pentamers and hexamers of TGT, but at the concentration of 1 mg/mL, this analysis indicated TGT to be monomeric. Gathering the results from DLS and *SEC*, it could be proposed that the chromatographic peak detected corresponds to the dynamic mixture of pentamers/hexamers. Regarding the secondary structure, also results are consistent with previous published studies [19] for a concentration similar to our CD analysis at 0.2 mg/mL TGT (reconstituted medicine diluted with water MilliQ), to which TGT is as monomers. At these conditions, TGT is predominantly random coil (45 %), with the smaller content for α-helix (8 %). With respect to the tertiary structure, reconstituted TGT (10 mg/mL) was characterized by a calculated C.M of 358.6 nm, which shifted to 363.4 nm for diluted TGT at 0.015 mg/mL, which indicated a more polar ambient in the surroundings of the tryptophan residue; this could be explained considering the self-association (pentamers/hexamer) of TGT at the concentration of 10 mg/mL and the monomeric stage of the peptide at the lower concentration of 0.015 mg/mL. As proposed by Philo et al. [19], the TGT association state of predominantly pentamer likely involves clustering of this single tryptophan residue which is more exposed to the solvent at the monomeric stage. Regarding TGT chemical structure, monomers (0.015 mg/mL) were characterized by an intact mass of 3751.7934 Da, which matches the theoretical mass described in the bibliography [38]. After subjecting reconstituted TGT (10 mg/mL) medicine sample to trypsin enzymatic digestion, three peptides (i.e. HGDGSFSDEMNTILDNLAAR, DFINWLIQTK and ITD) and eight modifications (deamidations, isomerizations and oxidations) were detected and identified. Nevertheless, the low abundance levels found (<2.5 %) for the PTMs meant that they could be present at these small quantities in the original medicine and also that they could have been induced during the enzymatic digestion protocol [32].

Results indicate that exposure to temperatures of 40 °C and 60 °C for 3 h induces aggregation in TGT samples, as occurs in other proteinaceous medicines [21,39]. This finding was corroborated by the results of the DLS and *SEC* analyses. *SEC* profiles demonstrated that aggregation increased proportionally in line with temperature (6 % and 14 % of the total areas in the chromatographic profiles attributed to HMWS for 40 °C

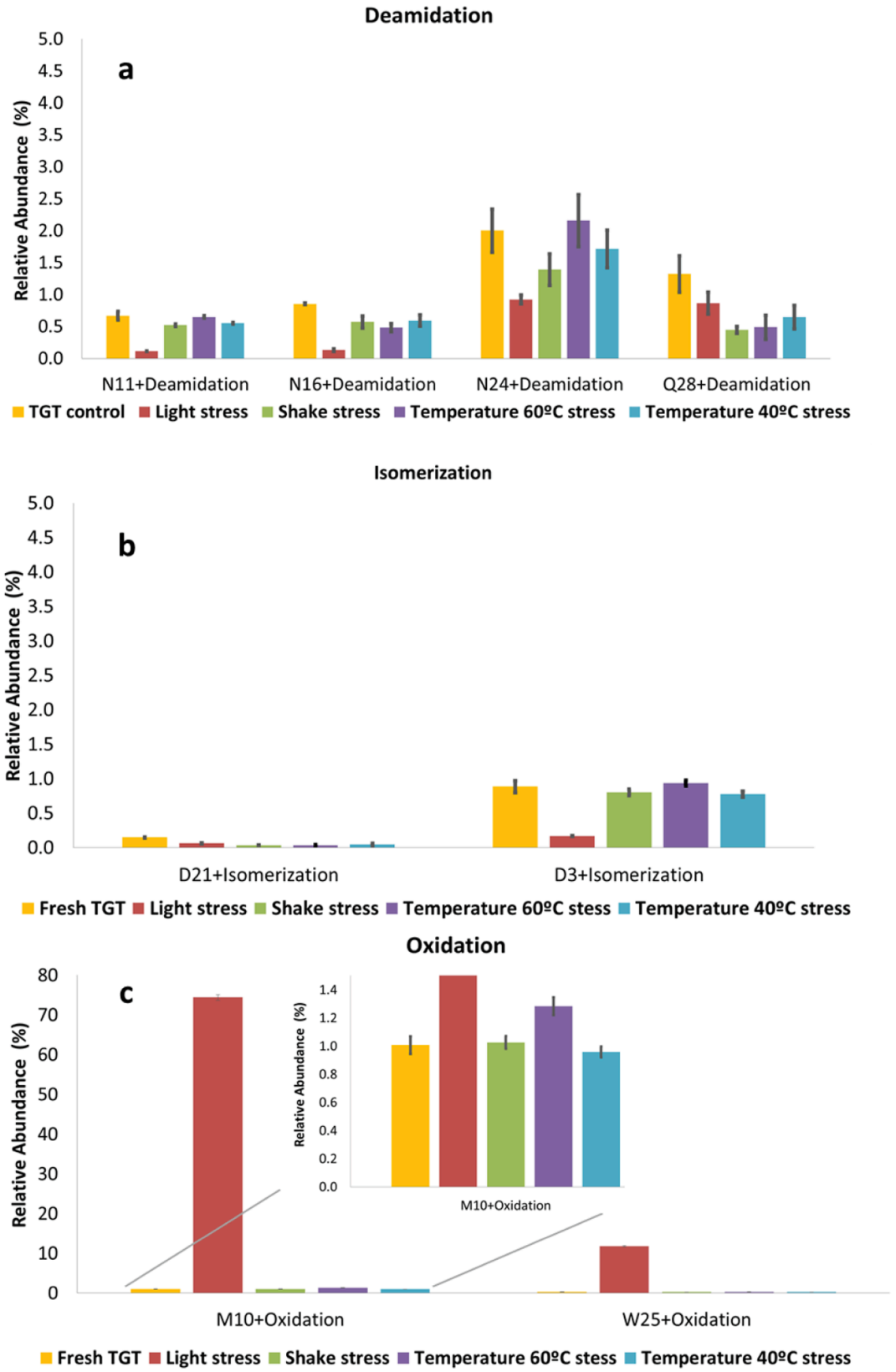

Fig. 8. Average relative abundance (n = 3) of identified PTMs (a. deamidation, b. isomerization and c. oxidation) in control and stressed samples of TGT (Revestive® reconstituted with water for injection for parenteral use at 10 mg/mL TGT). Error bars represent standard deviation (n = 3).

and 60 °C respectively) (Table 5). This aggregation or self-association was also suggested by DLS analysis, although no modifications were detected for sample subjected to 40 °C (attributed to the very small proportion, as indicated by *SEC*); D_h increases for samples subjected to 60 °C, assigned to heptamers of TGT formed at this temperature. These aggregations or self-associations were undetected by CD analysis, since at the concentration of 0.2 mg/mL (the higher that could be analyzes by CD) TGT is proposed to be as monomers. This result also indicated that the HMWS detected with the increase of the temperature were reversible (does not differ from the control sample). The CD spectra (Table 1 and Fig. 2) and the percentages of secondary structures (Table 2) remained unaltered, suggesting that this self-association did no promote changes in TGT monomer secondary structure that could be detected by CD. With respect to the tertiary structure, results by IP-FS indicated that this was similar to de control samples; only for the sample subjected to 60 °C, a very small change in the calculated C.M. was found (from 358.6 nm to 359.2 nm) that could be explained by the proposed presence of TGT hexamers in the solution. The intact mass analysis -performed at 0.015 mg/mL- showed one single mass corresponding to the intact mass of TGT without PTMs (3752.0919 Da); this result also indicates that the self-association promoted by the increase of the temperature up to 60 °C is reversible, since the aggregates (hexamers/heptamers) were not detected in the intact analysis by HRMS. No significant PMTs were detected in TGT from the temperature stressed samples with respect to the control sample, therefore indicating that the stress conditions investigated did no promote chemical modifications such as deamidation, oxidation etc.

The smooth shaking stress test caused a slight formation of HMWS of a similar size to those formed when the samples were subjected to 60 °C for 3 hours. This confirmed the findings of previous research in which some cases of peptide aggregation after stirring/shaking were reported [40]. Similarly, the aggregates were reversible, confirmed also by the intact analysis by detecting TGT monomers. No changes were detected in either the secondary or tertiary structures or in the intact mass or PTMs abundance levels regarding the control samples.

The 24 h accelerated light stress also led to aggregation in TGT but as a result of a degradation peptide process. It is well known that exposure to light induces aggregation in proteinaceous medicines [41], as can be seen in TGT, in which the aggregate population clearly increased during light exposure. Results from DLS and *SEC* suggested the presence of mixture of oligomers from pentamers up to decamers, therefore some of them were larger than those formed during the high temperature and shaking stress tests, this was also confirmed by the smaller retention times of the chromatographic peak detected d by *SEC*. In addition, light stress induces the formation of a population with a MW slightly higher to TGT monomers (65 % estimated by DLS) which was not detected in the control samples (TGT 10 mg/mL) since at this concentration TGT is proposed to be a dynamic mixture of pentamers/hexamers. The small peak detected at the higher retention times (12.91 min) could be attributed to this monomeric specie but the percentage of the area in the chromatogram is too small (5 %) and does not fit with the results obtained by DLS (65 %). As the *SEC* column was not calibrated for the estimation of the MW, no conclusion could be proposed by this peak. The secondary and tertiary structures of the TGT were modified after light stress, as manifested by the percentages of secondary structures (Table 2) and the C.M. (Table 3). It has previously been reported that light oxidizes tryptophan and methionine residues in biopharmaceutics [21,41,42]. In our research, this stress caused the oxidation of M10 and W25, reaching almost 80 % and 12 % of relative abundance respectively. Therefore, this percentage of W25 oxidized can explain the overall decrease of the fluorescence intensity in the spectra and the shift to higher C.M. could be attributed to a more exposed to the aqueous solvent of some part of the remaining no-oxidized tryptophan residue that could be at monomeric stage (as DLS results indicates). In addition, 3 oxidations were identified by intact MS analysis in the samples subjected to light stress, that could therefore indicate the oxidation of the methionine residue to methionine sulfoxide and sulfone.

5. Conclusions

Therapeutic proteins may be exposed to a wide range of stresses during handling in hospital settings. This study sought to assess the physicochemical stability/structure description of TGT (Revestive® reconstituted with water for injection for parenteral use at 10 mg/mL) after exposure to different physical stress conditions in order to establish the possible impacts of routine hospital manipulation. In this context, a comprehensive analysis of the TGT clinical solution was addressed. In agreement with previous published research on TGT drug product, we proposed that TGT after reconstitution from the lyophilized stage also self-associates, being this association reversible. Regarding the stress study, the results show that, even though TGT has a simple structure, its stability is affected by the application of certain stress stimuli. Light stress causes changes in the conformation of the peptide including aggregation, which is induced by chemical degradation (high % of M and W oxidations) that could compromise the safety and efficacy of the medicine. It is therefore important to prevent the medicine from being exposed to light. Our results also indicate that the medicine should be stored in a refrigerator, in that the higher the storage temperature the more prone the peptide is to form aggregates/self-associations and even oxidation products. Caution must also be taken when reconstituting the medicine given that shaking, especially over a long period, can also lead to increase the aggregation/self-associations. Exposure to light, high temperatures and prolonged shaking can therefore bring about changes in this drug which impair the efficacy and safety of the treatment, especially in the case of a strong light exposure. As expected, this research clearly demonstrates the fragile nature of biotechnological drugs and in particular of the peptide TGT.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejpb.2023.01.001.

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