Journal of Pharmaceutical and Biomedical Analysis 221 (2022) 115064

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.journals.elsevier.com/journal-of-pharmaceutical-and-biomedical-analysis

Tracking the physicochemical stability of teduglutide (Revestive®) clinical solutions over time in different storage containers

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ARTICLE INFO

Keywords:
Teduglutide
Long-term stability study
Hospital condition
Revestive®

ABSTRACT

Teduglutide, the active ingredient of the medicine Revestive® (5 mg), is a recombinant therapeutic peptide that mimics the effects of the endogenous glucagon-like peptide 2 (GLP-2). It stimulates intestinal growth, adaptation and function in patients with Short Bowel Syndrome who are dependent on parenteral nutrition. The Summary of Product Characteristics recommends immediate use of the reconstituted solutions and the discarding of any subsequent surplus. This study aims to carry out a long-term stability study that reproduces hospital conditions of use which provide sound evidence regarding the use of teduglutide surplus beyond the Summary Product Characteristics recommendations. We conducted a stability study of teduglutide solutions prepared from a 5 mg vial of Revestive®. Some of the solutions were stored in their original vial after reconstitution, while others were repackaged in plastic syringes to evaluate their physicochemical stability over time. For this purpose, we applied a set of previously validated analytical methodologies to evaluate the main critical quality attributes of teduglutide, i.e., primary (including post-tralational modifications), secondary and tertiary structures, aggregates, particulate, concentration and pH. The results indicate that the solutions maintain high physicochemical stability over time, regardless of the storage temperature (4°C or −20°C) or the storage container (vials or syringes). This research provides new data on the stability of Revestive® that will be of great value to hospital pharmacists. This comprehensive assessment of the physicochemical long-term stability of TGT has demonstrated that under the storage conditions and over the period studied here, the medicine maintains its quality, efficacy and safety profiles.

1. Introduction

Short bowel syndrome (SBS) is the most common cause of chronic intestinal failure in both children and adults. It is defined as a state of malabsorption resulting from massive resection of the small intestine. The most common causes of SBS in infants and young children are necrotising enterocolitis, congenital intestinal anomalies (atresia or gastroschisis), midgut volvulus, and long-segment Hirschsprung disease. In adults, the main causes are Crohn disease and radiation enteritis [1,2].

Treatment of SBS is based on a 3-pronged approach: nutritional support, pharmacological treatment and surgery. Nutritional support via parenteral nutrition (PN) or enteral nutrition is key to the growth and development of paediatric patients. Many pharmacological treatments are available to manage the symptoms and complications of SBS, including antisecretory agents, antibiotics, pancreatic enzymes, choleretic agents, and probiotics. More recently, growth factor and other trophic hormones have been used to improve intestinal adaptation. Bowel lengthening surgery (e.g., Bianchi or serial transverse enteroplasty procedure) or as a last resort, bowel transplantation, can improve patient quality of life [3].

Teduglutide (Gattex®, Revestive®) (TGT) is the only drug which has

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https://doi.org/10.1016/j.jpba.2022.115064
Received 12 July 2022; Received in revised form 12 September 2022; Accepted 15 September 2022
Available online 17 September 2022
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been approved for long-term treatment of SBS with beneficial effects observed in different clinical trial phases. Teduglutide is a recombinant human glucagon-like peptide 2 (GLP-2) analogue (a naturally- occurring peptide which is secreted primarily by the lower gastrointestinal tract) [4]. GLP-2 is released by enteroendocrine L cells in the distal ileum and proximal colon in response to the presence of unabsorbed luminal nutrients [5]. Reduced GLP-2 release following surgical resection contributes to decreased digestive and absorptive function of the remaining intestine. Exogenous GLP-2 (TGT) is thus used to stimulate intestinal growth, adaptation and function in SBS patients who are dependent on parenteral nutrition [6]. TGT ($C_{164}H_{252}N_{44}O_{55}S$) is expressed by a genetically modified strain of *E.coli*, made up of a single chain of 33 amino acids, with a molecular weight of 3752.1 Da [7]. It has no disulphide bonds, no glycosylation sites and no post-translational modifications. It differs from GLP-2 in that it has an Alanine to Glycine substitution in the second position of the N-Terminus. This substitution renders the peptide resistant to in vivo degradation by dipeptidyl peptidase IV and increases its half-life from just 7 min to approximately 2–3 h [8]. TGT is the active substance of two marketed medicines both of which were approved in 2012, namely Gattex® (Takeda Pharmaceuticals, Tokio, Japan), authorised by the U.S. Food and Drugs Administration (FDA), and Revestive® authorised by the European Medicines Agency (EMA) (Shire Pharmaceuticals, Dublin, Ireland).

Peptide-based medicines are proteinaceous in nature and are subject to instability issues. Stability studies are an essential regulatory requirement during the development, production and administration of proteinaceous medicines [9]. According to the ICH Q5C guideline, the objective of stability testing is to provide evidence on how the quality of a drug substance varies over time under different environmental conditions such as: temperature, light, humidity and a wide pH range [10]. In addition, stability testing allows scientists to establish a suitable retest period for a drug substance or a suitable shelf life for a drug product. It can also be used to recommend storage conditions for drug substances and drug products. However, in many cases the pharmaceutical industry establishes a 24-hour limit on the use of drugs once they have been reconstituted and conducts stability studies that demonstrate the stability of the drug over this short period. In fact, in the specific case of TGT, according to the SPC, it only remains stable for 3 h after reconstitution [7]. It is of paramount importance for hospital pharmacists to have well-documented data about the real stability of the reconstituted product over longer periods, which means that stability studies on biopharmaceuticals that reproduce hospital storage conditions are essential [11–13]. With these data, pharmacists can prepare several separate doses for one or more patients so as to optimise the use of the vial. These doses can be stored over a longer period, so avoiding the daily surplus of medicines in hospital pharmacies.

A large number of scientific publications have demonstrated that the use-by date for proteinaceous-based medicines could be extended beyond the manufacturer's indications. To this end, a proper biotherapeutic characterisation of the medicine must be conducted that can provide the same level of quality, efficacy and security as the manufacturer's indications. Long-term stability studies of biopharmaceuticals, above all monoclonal antibodies (mAbs) and Fc-fusion proteins, have been extensively reported in the literature [14–18]. All of these studies have confirmed that the use of these biopharmaceuticals can be extended beyond the manufacturer's indications under specific storage conditions. It is also widely accepted that several complementary analytical methods are required to fully assess the physicochemical stability of proteins given their structural complexity. This means that several different critical quality attributes (CQAs) must be studied i.e. primary, secondary and tertiary structure, post translational modifications (PTMs), oligomers and aggregates profile, drug substance quantification, etc. [19].

The objective of this study was to assess the physicochemical stability over 7 days of TGT (Revestive®) stored in its commercial vials after reconstitution (10 mg/mL) refrigerated (4ºC) and when stored in syringes of polypropylene (0.30 mm(30 G) x 8 mm, 0.3 mL vol.) under two different temperatures, refrigerated (4ºC) and frozen (−20ºC), over 28 and 45 days respectively. Therefore, the aim was to analyse the physicochemical stability of TGT clinical solutions beyond the expiry date appearing in the Summary of Product Characteristics (SPC). The SPC of Revestive® indicates physicochemical stability over 3 h at 25ºC following reconstitution. It does not provide any stability data regarding repackaged TGT in syringes [7]. With this in mind, several previously validated analytical methods were used to detect changes in physicochemical CQAs of TGT. The CQAs studied were particulates, oligomers, aggregates, PTMs (deamidations, isomerizations and oxidations), secondary and tertiary structures, peptide quantification and pH. According to the ICH Q5C, the study of these CQAs provides a broad range of information about the peptide and its long-term stability, and is essential to ensure its quality, efficacy and safety. To the best of our knowledge, this is the first long-term stability study conducted on a peptide-based medicine or TGT drug.

2. Design of the stability study

The stability study was performed according to an ad hoc work procedure that was based on several pharmaceutical quality assurance guidelines [11–13,20]. To this end, we assessed physicochemical quality attributes of TGT that were tested using a combined set of analytical methods described below in Section 3. All the methodologies were previously validated by subjecting freshly reconstituted samples of TGT to several forced degradation conditions that emulate situations that could arise during handling in hospital settings, namely exposure to high temperature (40ºC and 60ºC), light irradiation and shaking [21].

2.1. TGT clinical samples

Vials of Revestive® (Shire Pharmaceuticals Ireland Limited, Dublin, Ireland) containing 5 mg of the active ingredient (batch: T1903C1) were reconstituted with 0.5 mL ampoules of water for injection Meinsol® (Fresenius Kabi, Bad Homburg, Germany; batch 20PKF016) to obtain a final concentration of 10 mg/mL, according to the manufacturer's indications in the SPC [7]. Once reconstituted, the samples were prepared and stored at three different conditions. The original reconstituted vials were stored refrigerated at 4º (temperature range 4–8ºC) away from the light. Simultaneously, 100 μL aliquots of the reconstituted TGT solution were repackaged immediately in 0.3 mL Micro-Fine™ polypropylene syringes (0.30 mm(30 G) x 8 mm, 0.3 mL vol.) with a needle (BD medical, Franklin Lakes NJ, USA), batch 9154711C. Altogether, 3 vials and 18 syringes were stored at 4ºC, while 9 syringes were stored at −20ºC. All of these vials and syringes were protected from the light.

2.2. Stability studies over time

As mentioned earlier, in this research we used a set of previously validated analytical methods to assess the physicochemical stability of TGT. This procedure can be considered sufficiently exhaustive to establish a reliable stability-indicating profile for TGT. As stated in the international ICH guideline Q6B, biological tests can only be replaced by physicochemical tests if sufficient information about the higher order structures is obtained. With this in mind, we selected a range of spectroscopic techniques to assess the stability of the clinical solutions of TGT over time: far ultraviolet (UV) circular dichroism (CD) to study the secondary structures of TGT; intrinsic tryptophan fluorescence (IT-F) to study the conformation of TGT and dynamic light scattering (DLS) to analyse the formation of particulate in solution. These were combined with the following types of chromatography: size-exclusion high-performance chromatography with diode array detection (SE)HLPC-DAD to study aggregation; intact reverse phase ultra high-performance liquid chromatography with ultraviolet detection (intact(RP)UHPLC-UV) for

quantification of the peptide and peptide mapping reverse phase ultra high-performance liquid chromatography coupled with tandem mass spectrometry ((RP)UHPLC-(Orbitrap)MS/MS) to detect changes in the primary structure of TGT. The pH was also monitored over the period of study (see Table 1).

Three long-term storage periods were established. Three reconstituted vials were stored over a 7-day period and analysed on day 0 (D0, control), 24 h (D1), day 2 (D2), 3 (D3) and 7 (D7); repackaged TGT syringes stored at 4ºC for 28 days were analysed after 24 h (D1), and on days 2, 3, 7, 14 and 28 (D2, D3, D7, D14 and D28), and repackaged TGT syringes stored at − 20ºC for 45 days were analysed on D14, D28 and D45 (see Table 2). A total of 14 samples were studied in triplicate (three independent replicates). Each replicate was also analysed in triplicate (three instrumental replicates). The reconstituted vials at D0 were used as the control sample, with which all subsequent data were compared.

3. Materials and methods

3.1. Chemicals and reagents

Reverse-osmosis-quality water was purified with a Mili-Q station from Merck Millipore (Darmstadt, Germany). Trypsin (Trypsin Gold, Mass Spectrometry Grade) was obtained from Promega Corporation (Madison, USA). Ammonium bicarbonate (Bioultra, 99,5%), DL-dithiothreitol (DTT), Iodoacetamide (IAA) was obtained from Sigma-Aldrich (Wicklow, Ireland). Rapigest® SF surfactant was obtained from Waters Corporation (Milford, USA), Trifluoroacetic acid (TFA) from Scharlab S.L (Barcelona, Spain) and Acetic acid from Panreac (Barcelona, Spain). For the mobile phases of UHPLC, we used formic acid (FA) from Fisher Scientific (Geel, Belgium) and acetonitrile (ACN) from VWR International Eurolab S.L (Barcelona, Spain). Anhydrous disodium hydrogen phosphate and monohydrate monobasic sodium phosphate were supplied by Panreac (Barcelona, Spain) and Sigma-Aldrich (Missouri, USA).

3.2. 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 measurements. Solution samples were studied at the target concentration of 0.2 mg/mL. 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 1 mm path length was used throughout. The blank was first measured and subtracted from the samples and Means-Movement Smoothing was applied to all the spectra with Spectra Analysis software. Spectra were exported as ASCII files in order to estimate the secondary structure content using CDSSTR and CONTINL algorithms and the SP175 protein DataSet available at the Dichroweb online server [22].

Table 1
Stability study work outline.

	Clinical samples and conditions	Analytical techniques and attributes evaluated
Forced degradation	- Freshly reconstituted TGT samples (10 mg/mL) subjected to: light irradiation (250 W/m^2), heat (40 and 60ºC) and shaking (300 rpm) [21]	*Validated Methods:* - (RP)UHPLC-(HESI)MS/MS(Orbitrap) - Far UV Circular Dichroism spectroscopy (CD)
Stability over time	- TGT 10 mg/mL stored in vials at 4ºC (darkness) - TGT 10 mg/mL stored in syringes at 4ºC (darkness) - TGT 10 mg/mL stored in syringes at − 20ºC (darkness)	- Intrinsic Tryptophan Fluorescence spectroscopy (IT-FS) - Dynamic Light Scattering (DLS) - (SE)HPLC/DAD - (RP)UHPLC/UV *Critical Quality Attributes* - Primary structure - Secondary structures - Tertiary structure - Soluble particulate - Aggregates - Quantity -Deamidations -Isomerizations -Oxidations -pH

Table 2
Stability study design.

Time of analysis	Replicate 1	Replicate 2	Replicate 3	TOTAL (Sample)
Day 0	V[a]	V	V	1
Day 1	V, RS[b]	V, RS	V, RS	2
Day 2	V, RS	V, RS	V, RS	2
Day 3	V, RS	V, RS	V, RS	2
Day 7	V, RS	V, RS	V, RS	2
Day 14	RS, FS[c]	RS, FS	RS, FS	2
Day 28	RS, FS	RS, FS	RS, FS	2
Day 45	FS	FS	FS	1

[a] V= vial.
[b] RS= Refrigerated syringe (4°C).
[c] FS= Frozen syringe (-20°C).

3.3. Intrinsic tryptophan fluorescence spectroscopy

Measurements were carried out on a Cary Eclipse spectrofluorometer (Agilent, Santa Clara, CA, USA). Excitation wavelength was set at 298 nm and emission was recorded from 300 to 400 nm. Solution samples were diluted to 15 ppm before measuring at room temperature in a 45 µL fluorescence quartz cuvette. Spectra were recorded at a scan speed of 600 nm/min and 3 measurements were taken, each with a total of 5 spectral accumulations. Excitation and emission slits were set at 20 and 10 nm respectively. The spectra centre of mass (C.M.) was calculated for each spectrum within the 318–400 nm interval.

3.4. Dynamic light scattering (DLS)

DLS readings were carried out on a Zetasizer Nano-ZS90 (Malvern, UK). A 12.5 µL volume thermostatted quartz cuvette with a path length of 1.5 nm was used throughout. The cuvette was cleaned thoroughly before every measurement and a total of 100 readings, with an acquisition time of 5 s each, were taken per measurement at 20ºC. HD±SD of the solution samples was averaged and analysed over time. The Z average and PDI values of the samples were also analysed over time.

3.5. Size-exclusion high-performance chromatography with diode array detection (SE/HLPC-DAD)

The analysis was carried out using an Agilent 1100 system equipped with a degasser, autosampler, quaternary bomb and photodiode array detector (DAD), (Agilent Technologies, USA).

The size exclusion analyses were evaluated in an Agilent BioSEC-5 column (300 mm×4.6 id., 5 µm particle size, 150 Å wide pore) (Agilent Technologies, USA). The peptide was eluted using isocratic mode for 20 min with a flow rate of 0.3 mL/min. The mobile phase was composed of 150 mM of phosphate buffer pH 7.0. The UV–vis range recorded was 190–400 nm, with a spectra data point every 2 nm. Chromatograms were registered at 214 nm using 360 nm as the reference band.

3.6. Intact reverse phase ultra high-performance liquid chromatography with ultraviolet detection (intact(RP)UHPLC-UV)

This was performed using a chromatograph (Ultimate 3000, Thermo Scientific, Waltham, MA, USA) equipped with two ternary pumps, a degasser, an autosampler, a thermostatted column compartment, and a multiple-wavelength detector (MWD-3000(RS)UV–Vis detector).

An Acclaim Vanquish C18, 2.2 mm, 2.1 mm × 250 mm column (Thermo Fisher Scientific, Waltham, MA, USA) was used for the chromatographic separation. The flow rate was 0.3 mL/min and 5 μL of samples were injected into the column. The column temperature was 25ºC. 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

3.7. Tryptic digestion

In this case, we applied the same protocol as in our previous research [21]. The tryptic digestion was performed in 0.5 mL Eppendorf tubes, in which 100 μg of TGT were diluted to 2 mg/mL in buffer digestion (NH_4HCO_3, 50 mM at pH 7.8). The peptide was denaturalised with the addition of 50 μL of Rapigest® surfactant (0.1% reconstituted in buffer digestion) and was incubated at 80ºC 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 37ºC and subsequent alkylation with IAA 60 mM at 25ºC 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 37ºC. The reaction was quenched with 10 μL of TFA (25% in water) and incubated for 30 min at 37ºC. 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 HPLC amber vials. The tryptic peptides were separated and monitored by LC-MS/MS (the chromatographic method is described below in Section 3.8 "*Peptide mapping reverse phase ultra high-performance liquid chromatography coupled with tandem mass spectrometry ((RP)UHPLC-(Orbitrap)MS/MS)*". Each sample replicate was digested in triplicate.

3.8. Peptide mapping reverse phase ultra high-performance liquid chromatography coupled with tandem mass spectrometry ((RP)UHPLC-(Orbitrap)MS/MS)

The chromatograph used for the separation and identification of the tryptic digestion peptides by (RP)UHPLC-(Orbitrap)MS/MS was the same as used in Section 3.6. "*Intact reverse phase ultra high-performance liquid chromatography with ultraviolet detection (intact(RP)UHPLC-UV)*". It was coupled in line to a Q-Exactive Plus mass spectrometer (Thermo Scientific, Waltham, MA, USA). Ionisation was performed using a heated electro spray ionisation (HESI) source.

The analyses were performed using a binary gradient of 0.1% (v/v) formic acid in water (mobile phase A) and 0.1% (v/v) formic acid in acetonitrile (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. Gradient was kept at 80% B for 4 min, and then shifted to 2% B in 0.5 min. Lastly, 2% B was kept for 5 min for column reconditioning. Column temperature was kept at 25ºC throughout and flow rate was 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 ºC.The MS method consisted of full positive polarity MS scans at 70,000 resolution setting (at m/z 200) with the mass range set to 200–2000 m/z and AGC target value of 3.0×10^6 with 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), AGC target value of 1.0×10^5, isolation window set to 2.0 m/z and a maximum IT of 200 ms.

3.9. pH monitoring

The pH of the clinical solutions was monitored throughout the study period using a pH metre (XS instruments, Italy) equipped with a mini electrode (Hamilton, Switzerland).

3.10. Data processing

The intact peptide quantification was performed using Xcalibur QualBrowser 4.0 for signal integration. The Statgraphics Centurion 18 software package was used for obtaining the calibration curves and for the statistical data processing (ANOVA). The peptide mapping data processing, quantitation and identification were performed on BioPharma Finder 3.1 software (Thermo Scientific) using the parameters summarised in Table S1. The secondary structures content was estimated using CDSSTR [23] and CONTINL [24] algorithms and the SP175 protein DataSet [25] available at the Dichroweb website [26].

4. Results

4.1. Far UV Circular Dichroism (CD) Spectroscopy

The secondary structures of TGT were characterised by Circular Dichroism spectropolarimetry in the Far-UV region. Visually, the representative TGT spectrum at D0 (control) has two minima at 201 and 227.4 nm respectively, and a maximum at 215.6 nm (Fig. 1). TGT at the target concentration of 0.2 mg/mL has the typical CD spectrum associated with a majority of unordered structure (47 ± 3.6%) and β sheet (25.3 ± 1.5%), followed by turns (17%) and α-helix (10 ± 4.4%) according to [21].

Representative Far-UV CD spectra of the TGT solution samples are shown in Fig. 1. The spectra remained constant regardless of the container during the stability study period; i.e. 7 days in the case of the vials stored at 4ºC, 28 days in the syringes stored at 4ºC and 45 days in the syringes stored at − 20ºC. The estimations of secondary structure content are shown in Supplementary Material Fig. S1, which charts these percentages over time in all the TGT solution samples. These results confirm that the secondary structure content remained unchanged over time.

4.2. Intrinsic tryptophan fluorescence spectroscopy (IT-FS)

IT-FS was employed to characterise the structure of the therapeutic peptide. This produced a C.M. value at D0 (control) of 359 nm (Table 3). This high value indicates that the single tryptophan appearing in the structure of TGT is highly exposed to the solvent, as happens in small peptides with no tertiary structure.

The C.M. values of the IT-F spectra for the TGT solution samples were calculated over the various stability study periods (Table 3). The C.M remained constant over time in all the clinical samples, regardless of the container or the storage temperature.

4.3. Dynamic Light Scattering (DLS)

TGT particulate with a hydrodynamic diameter of up to 10 μm was characterised by DLS. This technique was also used to monitor the appearance of the particulate in solution. TGT at D0 (control) showed a single population in the size distribution by volume with an average (hydrodynamic diameter) HD±SD of 4.01 ± 0.11 nm (Fig. 2), which represented 100% of the population; a Z average of 14.1 ± 7.9 and a polydispersity (PdI) of 0.39 ± 0.12. This indicated that the control TGT solution samples were moderately polydisperse.

As regards the stability of the TGT solution over time, there were no

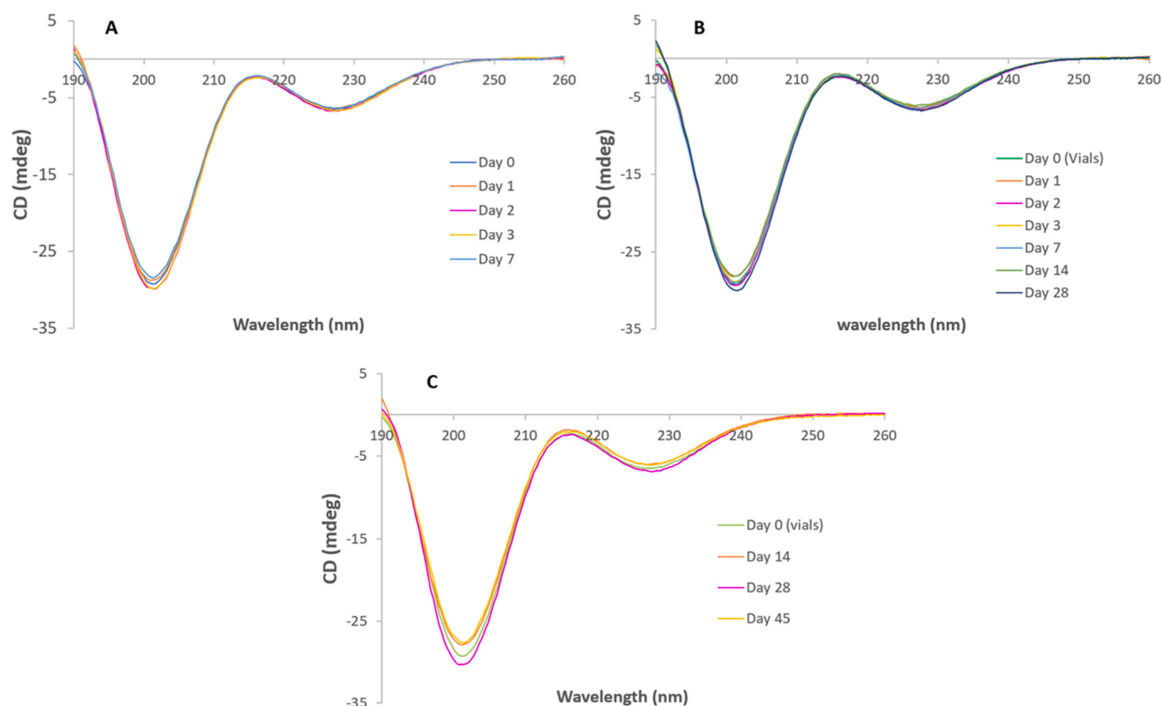

Fig. 1. : Average Far UV CD spectra over time of (A) TGT solution samples stored in vials at 4ºC for 7 days; (B) TGT solution samples stored in syringes at 4ºC for 28 days and (C) TGT solution samples stored in syringes at − 20ºC for 45 days.

Table 3
Spectral C.M. of TGT clinical solutions over the stability study period.

Day	Vials (4ºC)	Refrigerated syringes (4ºC)	Frozen syringes (−20ºC)
0 (control)	359	–	–
1	359	359	–
2	359	359	–
3	359	359	–
7	359	359	–
14	–	359	359
28	–	359	359
45	–	–	359

significant differences in the HD values in any of the samples over time; the size distribution by volume of the main population remained unchanged, representing 100% in all cases (Fig. 2). However, the ANOVA analysis applied to the Z average values revealed a significant effect in the solution samples stored in syringes at − 20ºC on day 45 (245.8 ± 45.8 nm). This could indicate the appearance of particulate, albeit in very small proportions (see Supplementary Material Fig. S2). PdI values were also studied by ANOVA, and no significant effects were obtained in any of the solution samples, except for the syringes stored at − 20ºC on day 28 (0.992 ± 0.12) and day 45 (0.975 ± 0.12) (see Supplementary Material Fig. S3).

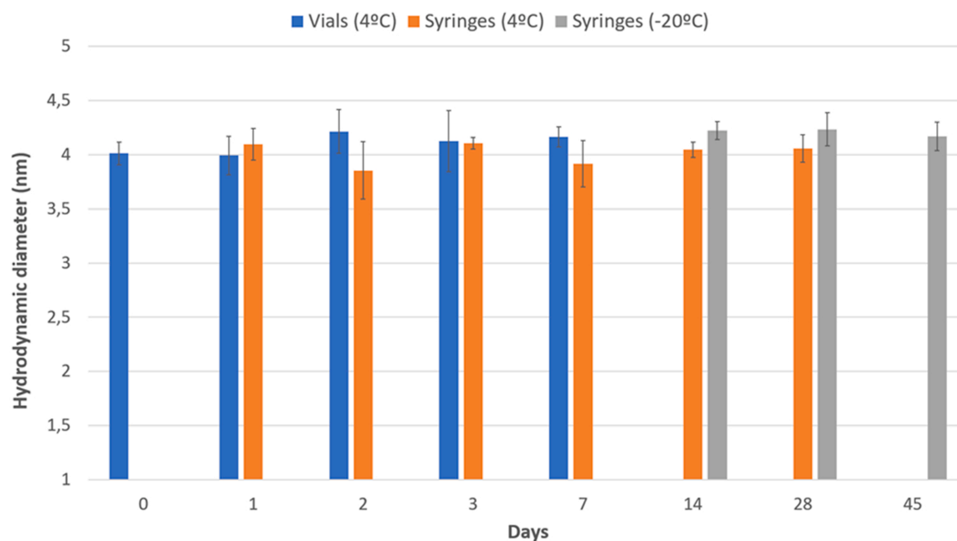

Fig. 2. : Evolution of the average HD±SD of the TGT solution samples over time. In blue, solution samples stored in vials for 7 days; in orange, solution samples stored in syringes for 28 days; and in grey solution samples stored in syringes at − 20ºC for 45 days in grey. The standard deviation values are included as an error bar,.

4.4. Size-exclusion high-performance chromatography with diode array detection (SE/HLPC-DAD)

SE/HPLC-DAD was used to detect aggregation (dimers, trimers, etc.) on TGT (10 mg/mL) solution samples. A representative chromatogram corresponding to D0 (control) was chosen as the control sample. As shown in Fig. 3A, the control chromatogram for TGT was characterised by a single peak: 12,605 ± 0.026 min of retention time, which in our previous research was found to be associated with the monomer [21], and an area under the peak of 4531.55 ± 2.97.

Fig. 3 shows representative SEC chromatograms for TGT solution stored in vials (Fig. 3A), in syringes stored at 4ºC (Fig. 3B) and syringes stored at − 20ºC (Fig. 3C) during the stability study. These chromatographic profiles remained unchanged over the study period, in terms of both the elution time and the area under the peak (see Supplementary Material, Table S2). No new aggregations or fragmentations were detected on any of the test days. Our analysis of all the chromatographic profiles clearly indicates high stability and a high degree of similarity in the solutions over the study period.

4.5. Intact reverse phase ultra high-performance liquid chromatography with ultraviolet detection (intact(RP)UHPLC-UV)

The method used here to study the concentration of TGT using RP/-

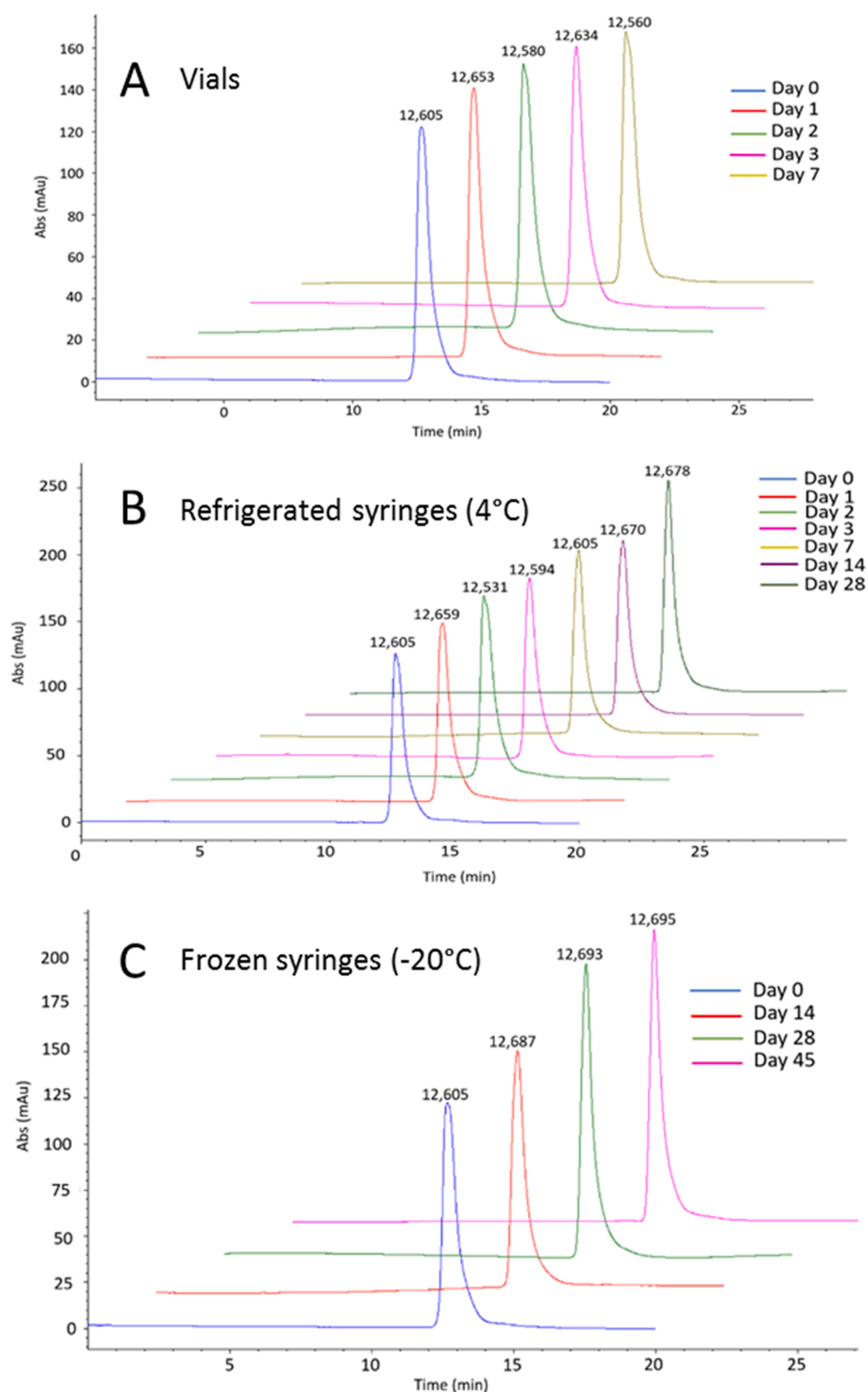

Fig. 3. : Representative chromatograms of TGT solution samples (10 mg/mL) obtained from (SEC)HPLC-DAD): (A) stored in vials (4ºC), (B) stored in refrigerated syringes (4ºC) and (C) stored in frozen syringes (−20ºC) over the stability study.

UHPLC-UV was developed in a previous paper in which it was also explained and validated (for quantification purposes). In addition, the method was validated for detecting degradation products in TGT [21]. A summary of the figure of merits of the instrumental method validation are showed in Table 4.

Aliquots of TGT (10 mg/mL) stowed in vials and syringes (stored at 4 °C and −20 °C) for 45 days were analysed. Each solution sample was analysed in triplicate. The calibration line was performed daily in order to avoid systematic errors in the estimation of TGT concentration. In addition, ANOVA analyses were applied in order to detect significant differences between the calibration lines. The results demonstrated a high degree of stability in which concentration was unaffected by the storage conditions. Fig. 4 shows the evolution of TGT concentration. The mean concentration estimated at D0 (9.8 ± 0.3 mg/mL, n = 3) was taken as a reference and compared with the concentration levels calculated over the course of the stability study. No significant changes in the concentration were observed during the study, indicating that the concentration of TGT remained constant over the 14 days stored in refrigerated vials (4ºC), 28 days stored in refrigerated syringes (4ºC) and 45 days stored in frozen syringes (−20ºC) with no adsorption on the wall of the container.

4.6. Peptide mapping reverse phase ultra high-performance liquid chromatography coupled with tandem mass spectrometry ((RP)UHPLC-(Orbitrap)MS/MS)

The primary structure of TGT (10 mg/mL) was characterised at D0 (control) using peptide mapping analysis by ((RP)UHPLC-(Orbitrap) MS/MS). The PTMs regarded as critical quality attributes (CQAs) i.e. deamidations, isomerizations and oxidations were analysed. These CQAs can have a negative impact on the quality, safety and efficacy of therapeutic proteins [27]. TGT is a small peptide made up of 33 amino acids, from which 3 peptide fragments were obtained by enzymatic digestion. One hundred percent sequence coverage was attained for all the samples under investigation (see Supplementary Material, Fig.S4). A total of 8 PTMs were reported in TGT at D0. Of these, 4 sites were identified as containing deamidations, 2 sites contained oxidations and 2 sites contained isomerizations, in line with [21]. The maximum relative abundance value obtained in any PTM was 2.5%. As reported in previous research, these small percentages of PTM might arise during the enzymatic digestion process, rather than during the period in which long-term stability was monitored [21].

Fig. 5 shows the average relative abundance of the reported PTMs in TGT stored in vials. No new deamidation sites were found during the period of study compared to the control (D0). The relative abundances of deamidations in Asn11 and Asn16 remained stable over the 7 days of

Table 4
Figure of merits of the intact (RP)UHPLC-UV [21] method validation.

Parameter	value
Slope (AU[1]/ (mg/L))	7.90×10^3
Intercept (AU[1])	-1.88×10^4
Linear Interval (mg/L)	5–25
Limit of detection (mg/L)	0.64
Limit of quantification (mg/L)	2.14
Relative error (at 15 mg/L)	4.7
Interday precision (Relative standard deviarion, (%))	0.64
Repeatability (Relative standard deviarion, (%))	2.14

[1] Arbitrary units.

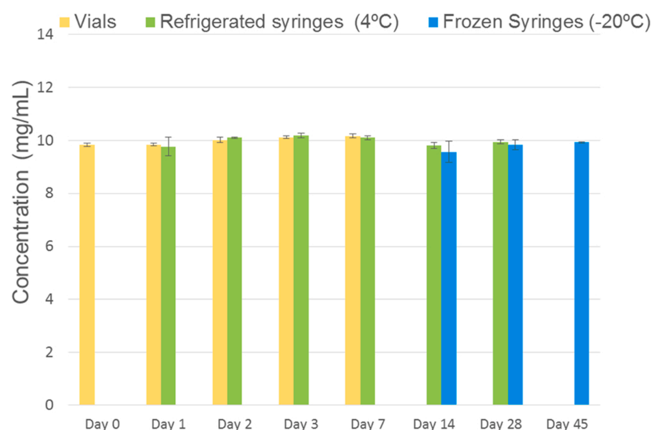

Fig. 4. Evolution of the TGT solution concentration over time. In yellow, solution samples stored in vials for 7 days; in green, solution samples stored in syringes for 28 days; and in blue solution samples stored in syringes at 4ºC for 28 days. The standard deviation values are included as an error bar.

study. As regards the Asn24 and Gln28 residues, slight increases in the percentages of deamidations were found from D1, although this was never more than 0.5%. For their part, the relative abundances of isomerizations (Asp3, Asp21) and oxidations (Met10 and Trp25) remained unaltered in the TGT solution stored in vials throughout the study period. Despite the fact that several PTMs were detected, the abundance values were under 3% in all cases. This suggests that these PTMs may have appeared during the enzymatic digestion process [28].

The TGT solution stored in syringes at 4ºC for 28 days or at − 20ºC for 45 days (see Supplementary Material, Fig. S5 and Fig. S6) behaved in a very similar way to the TGT solution stored in vials. A very slight, progressive percentage increase (of less than 0.5%) in Asn24 and Gln28 residues was observed in the samples, an increase that was almost inappreciable in the solutions stored in syringes. No changes were detected in the percentages of isomerization or oxidation in TGT in the syringes stored at 4ºC and − 20ºC, over their respective study periods.

4.7. pH monitoring

The pH is one of the key parameters to screen during the development and storage of biopharmaceuticals, as it has a direct influence on the protein net's charge.

The pH of TGT (10 mg/mL) stowed in vials and syringes (stored at 4 °C and −20 °C) were studied in triplicate for 45 days. The pH of freshly reconstituted TGT solution was in the range 7.44 ± 0.04. The pH remained stable over time in all the solutions (stored in vials and in frozen and refrigerated syringes) and was within the pH range for freshly reconstituted TGT (Fig. 6).

5. Discussion

A set of previously validated analytical methodologies [21] were selected for a comprehensive analysis of the stability of TGT (10 mg/mL) in real hospital conditions of use –vials and syringes– stowed under different storage conditions (vials refrigerated at 4ºC for 7 days, syringes refrigerated at 4ºC for 28 days and syringes frozen at −20ºC for 45 days). These conditions were tested in order to assess the stability of TGT solutions prepared in advance under sterile conditions in hospital facilities in order to prepare several doses for one or more patients individually. This would enable doses to be stored for longer periods, so avoiding the surpluses generated daily in the hospital pharmacy, which are normally thrown away. From a clinical perspective, the preparation of individualised doses under sterile conditions could increase the safety and improve the adherence to treatment, thereby enhancing the drug cost efficacy. In this study, analytical stability was assessed by measuring the

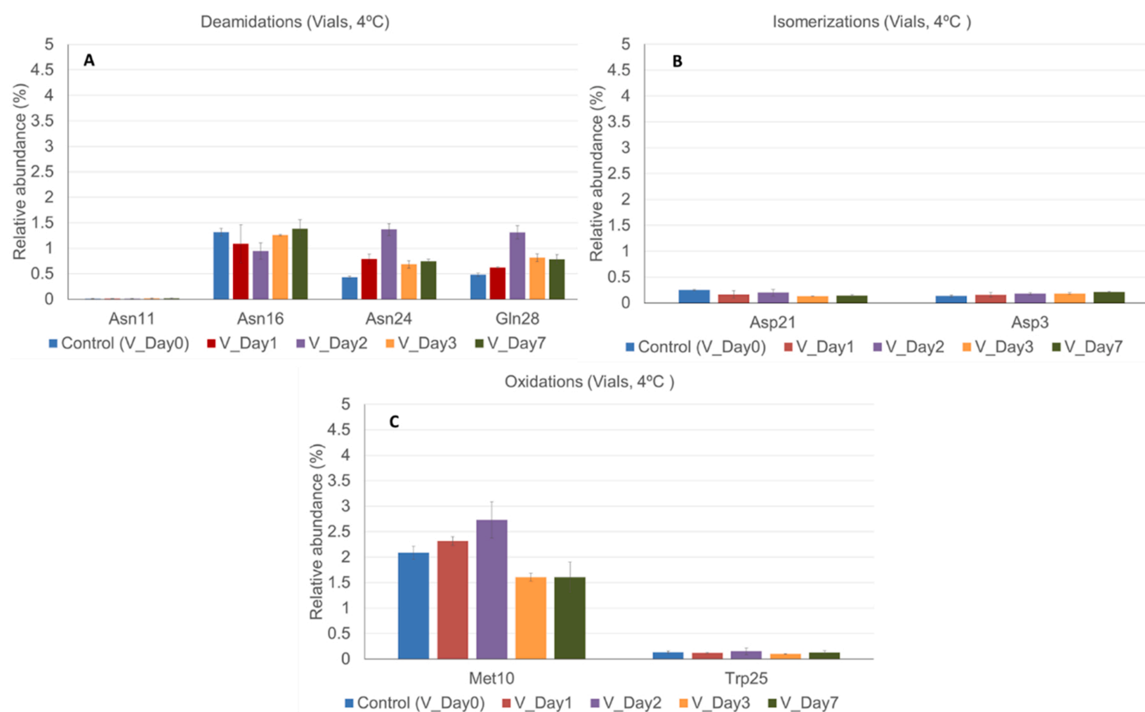

Fig. 5. Average relative abundance (n = 3) of the PTMs (A deamidation, B. isomerization and C. oxidation) identified in TGT (Revestive®) samples stored in vials (at 4ºC) for 7 days. Error bars represent standard deviation (n = 3).

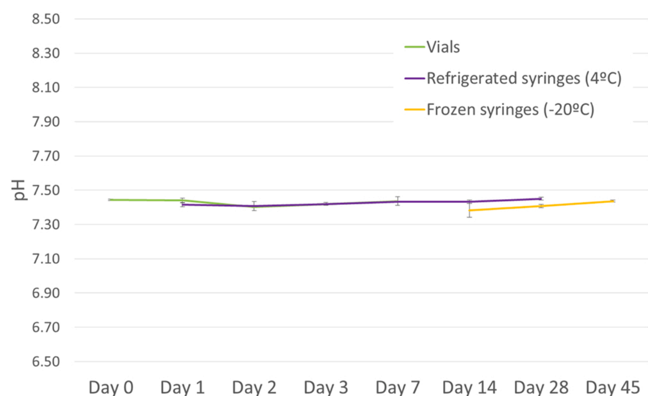

Fig. 6. Evolution of pH in TGT solutions over time. In green, solution samples stored in vials for 7 days; in purple solution samples stored in syringes for 28 days; and solution samples stored in syringes at 4ºC for 28 days in yellow. The standard deviation values are included as an error bar,.

values of the main critical quality attributes (CQAs) of the TGT samples over the aforementioned periods.

Aggregation was evaluated using two analytical methods: SEC for the analysis of oligomers and DLS for analysing larger soluble particulate populations up to 10 µm. The results indicated that no changes had taken place in the solutions, which were unaffected by the different storage and temperature conditions. However, in the solutions stored in syringes at − 20ºC, the ANOVA statistical test revealed that Z-average values were slightly higher on day 45 and Pdi values on days 28 and 45. This could indicate a tendency towards the formation of new particulate, albeit in negligible proportions. Although the ANOVA test revealed slight differences (in the Z-average and the PdI values) from day 28 in frozen syringes, it was not considered a stability issue given the results obtained by SEC and by DLS size distribution by volume (no significant differences between the control and the samples in the long-term stability study).

TGT is structurally very simple and has no tertiary structure [21]. However, changes in the spectral centre of mass were detected during stress testing, which mean that a study of its conformation would be useful. The spectral centre of mass remained highly constant over the three stability study periods, regardless of the container and the storage temperature. This confirms that the tryptophan environment is not affected over time, maintaining its native conformation. The secondary structure of the peptide was assessed by CD spectroscopy. In all the TGT solutions, it was confirmed that the secondary structure remained stable over the three different stability periods. The CD spectra features of the TGT remained unaltered over time in all the solutions. Moreover, the estimation of the secondary structure content indicated a high percentage of random coil and β sheet which remained the same in all cases. The abundance values for the PTMs (oxidations, deamidations and isomerizations) detected in TGT were quantified by a peptide mapping-(RP)UHPLC-(Orbitrap)MS/MS) method. As regards deamidations, the quantification revealed that the abundance value was less than 3% in all cases. A progressive slight percentage increase (less than 0.5%) in Asn24 and Gln28 residues in the samples stored in syringes at 4 and − 20ºC. Nonetheless, given the low percentages observed, these changes were not considered significant. With regard to oxidations and isomerizations, no increases in the abundance values were observed in any of the clinical samples assessed. The peptide content was evaluated using the intact (RP)UHPLC-UV method. It remained stable in all the clinical solutions, regardless of the storage conditions. pH was also shown to be unaffected by storage conditions and remained unchanged. This indicated that no degradation or bacterial growth took place in any of the solutions.

The ICH guideline Q6B states that biological tests can only be replaced by physicochemical tests if sufficient information about the higher order structures is obtained. In this case, given the simplicity of the peptide, functional studies were unnecessary. In this research, the structure of TGT was comprehensively analysed by assessing its physicochemical critical quality attributes. We consider this an appropriate way of establishing a proper stability-indicating profile for TGT [20].

6. Conclusions

In this research, we assessed the stability of TGT clinical solutions by applying a set of analytical methodologies that had previously been validated for detecting changes in the physicochemical properties of TGT. To this end, various CQAs were evaluated over time in clinical solutions prepared and stored in either vials (during 7 days at 4ºC) or syringes (during 28 days at 4ºC and 45 days at −20ºC). These CQAs remained stable over time with only minor changes in the spectroscopic and chromatographic profiles, indicating great physicochemical stability of the solutions, regardless of the container and/or storage temperature. As far as we know, this is the first time that the in-use stability of the therapeutic peptide teduglutide (Revestive®, 5 mg) has been studied. This comprehensive assessment of the physicochemical stability of TGT has demonstrated that under the storage conditions and over the period studied here, the medicine maintains its quality, efficacy and safety profiles. This indicates that patients (for example, paediatric patients) might be able to use supposedly single-use vials on multiple occasions. The preparation of individualised syringes under sterile conditions in pharmacy departments might also be feasible. This would result in considerable economic savings. The preparation in advance of individualised doses in syringes would also enhance safety, by preventing microbiological contamination and ensuring correct dosing.

Founding

Fundación Andaluza de Farmacia Hospitalaria (Spain); Hospital Paediatrics Pharmacy Unit of the Hospital Vall d′ Hebron (Barcelona, Spain); Junta de Andalucia (Spain), Universidad de Granada (Spain).

CRediT authorship contribution statement

Raquel Pérez-Robles: Conceptualization, Formal analysis, Investigation, Writing-Original Draft, Writing-Review and editing, Visualization, Supervision. **Jesús Hermosilla**: Conceptualization, Formal analysis, Writing-Original Draft. **Natalia Navas:** Conceptualization, Methodology, Formal analysis, Writing - review & editing, Supervision, Funding acquisition, Project administration, Resources. **Susana Clemente-Bautista:** Conceptualization, Investigation, Resources, Writing-Review and editing, Visualization. **Inés Jiménez-Lozano:** Conceptualization, Investigation, Resources, Writing-Review and editing, Visualization. **Maria Josep Cabañas-Poy:** Conceptualization, Investigation, Resources, Writing-Review and editing, Visualization. **Julio Ruiz-Travé:** Formal analysis, Writing-Original Draft. **María Amparo Hernández-García:** Formal analysis, Writing-Original Draft. **Jose Cabeza:** Resources, Funding acquisition. **Antonio Salmerón-García:** Conceptualization, Formal analysis, Resources, Funding acquisition, Visualization, Investigation.

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.

Acknowledgements

This study was founded by Fundación Andaluza de Farmacia Hospitalaria (Spain), TEC01 from Ibs. Granada and FQM 118 from University of Granada research groups. Raquel Pérez-Robles currently holds a postdoctoral position granted by the Junta de Andalucía, Spain (ref: DOC_01694). Jesús Hermosilla is currently benefiting from a research contract (Proyect ref: P20_01029) from the Junta de Andalucía (Spain) and European Regional Development Funds. The authors would like to thank the Hospital Paediatrics Pharmacy Unit of the Hospital Vall d′ Hebron (Barcelona, Spain) and the Hospital Clinico San Cecilio (Granada, Spain) for kindly supplying the medicine samples. Funding for open access charge: Universidad de Granada / CBUA.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2022.115064.

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