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Caprylate/chromatography process to produce highly purified tetanus immune globulin from human plasma

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Abstract

While tetanus toxoid vaccination has reduced the incidence of tetanus in the developed world, this disease remains a substantial health problem in developing nations. Tetanus immune globulin (TIG) is used along with vaccination for prevention of infection after major or contaminated wounds if vaccination status cannot be verified or for active tetanus infection. These studies describe the characterisation of a TIG produced by a caprylate/chromatography process. The TIG potency and presence of plasma protein impurities were analysed at early/late steps in the manufacturing process by chromatography, immunoassay, coagulation and potency tests. The caprylate/chromatography process has been previously shown to effectively eliminate or inactivate potentially transmissible agents from plasma-derived products. In this study, the caprylate/chromatography process was shown to effectively concentrate TIG activity and efficiently remove pro-coagulation factors, naturally present in plasma. This TIG drug product builds on the long-term evidence of the safety and efficacy of TIG by providing a product with higher purity and low pro-coagulant protein impurities.

Introduction

Tetanus is an acute bacterial infection caused by the obligate anaerobe, *Clostridium tetani*. While the bacterium itself cannot grow in the presence of oxygen, it produces spores which are resistant not only to exposure to air, but also to heat and many antiseptics. These spores can survive for years in the environment. Tetanus spores are found worldwide but are more common in densely populated areas and those with rich soil containing significant amounts of organic material. Other reservoirs for the spores include the gastrointestinal tracts of animals and some humans. The spores enter the body through an open wound and germinate under favourable anaerobic conditions. The growing bacteria produce a potent tetanus toxin, a neurotoxin that can be fatal if the infection is not promptly treated [1, 2].

While the availability of the tetanus toxoid vaccine has greatly reduced the incidence of tetanus in developed countries, tetanus remains a significant public health problem in developing nations [1]. In these parts of the world, the most common tetanus infections are perinatal in newborns and unvaccinated mothers. The World Health Organization estimated that worldwide 34 000 neonates died from tetanus in 2015. However, this number represents a substantial reduction in deaths (96%) when compared to 787 000 deaths in 1988 [1].

Although tetanus is not routinely a threat in developed countries, large-scale natural disasters can increase the danger due to the nature of injuries suffered, lack of information on vaccination status and the potential limitations on medical care and supplies (e.g. tetanus vaccine and tetanus immune globulin (TIG)). This increased threat during natural disasters is exacerbated in less developed countries due to already limited medical resources [3].

Another factor that could potentially increase the threat of tetanus infection is the increase in the elderly population. While it is routine for young people to receive the initial tetanus vaccination series, it is not uncommon for adults to neglect the 10-year boosters needed to maintain immunity. This fact along with the decreased immune response to vaccinations in the aged population are likely contributors to the greater lethality of tetanus infections in people over the age of 65 years [4–7].

In the USA, the number of deaths due to tetanus has been declining since 1900 and the nationwide incidence has been declining since cases were first systematically recorded in the 1940s. Death rates have decreased from 2.5/100 000 in 1900 to 0.01/100 000 in 2016 [8]. Just two deaths from tetanus were reported in 2017. Case rates decreased from about 0.5/100 000 in the mid to late 1940s to only 26 total cases in 2019 (corresponds to 0.008/100 000) [9]. The declines in tetanus in the USA and worldwide can be attributed to availability of the tetanus toxoid vaccine, improvements in wound care and availability of TIG for prophylaxis and

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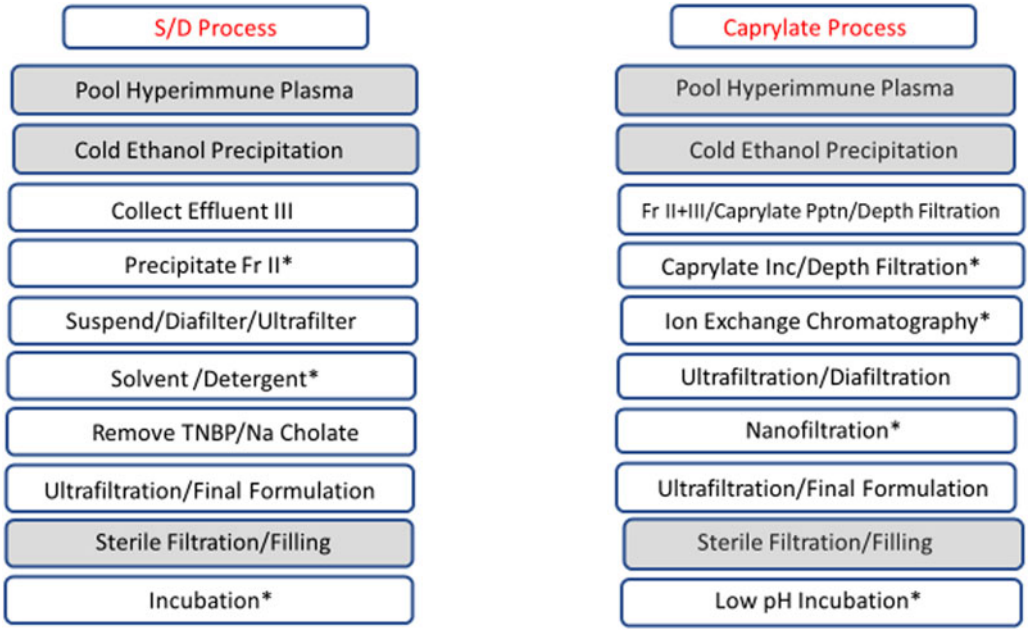

Fig. 1. Flow diagrams for the solvent/detergent (S/D) and caprylate/chromatography processes. The grey bars denote steps that are common to both processes and the asterisks (*) mark steps that have been validated to deactivate and/or remove blood-borne pathogens. Fr,fraction; TNBP,tri-n-butyl phosphate; Pptn,precipitation; Inc,incubation. This figure was modified from Woznichak *et al.* [24].

treatment of tetanus. Prompt identification of possible tetanus cases and immediate treatment with TIG and vaccination with tetanus toxoid may help decrease the severity of the disease [1, 10].

TIG is used in combination with the vaccine in the prophylaxis of tetanus infection when a wound cannot be classified as clean or minor and the vaccination status of the patient is unknown or if the patient has received less than the three-dose primary series of tetanus toxoid vaccinations. The wounds for which TIG is recommended include contaminated wounds, punctures, avulsions and wounds from missiles, crushing or frostbite [3, 5]. In addition, TIG can also be used in the treatment of active tetanus [11–15]. Passive immunisation via administration of antibodies has been successfully used for the treatment of many different infectious diseases [16, 17].

The product described in this paper (TIG-caprylate/chromatography process: TIG-C) was manufactured using a new manufacturing process which involves treatment with caprylate and purification by chromatography [18–20]. While the safety profile of TIG manufactured using the previous solvent detergent purification process is excellent, some procoagulant factors were retained in the products [21]. The new caprylate/chromatography was developed to reduce the procoagulant activity in the resulting TIG-C product. The analytical characteristics of TIG-C were determined and will be described in the following sections. This caprylate/chromatography process has been used for the production of polyvalent immunoglobulins for intravenous (IGIV) and subcutaneous use (IGSC) (Gamunex-C® and Xembify®, Grifols, Research Triangle Park, NC, USA), anti-SARS-CoV-2 immune globulin [22], anti-Ebola virus immune globulin [23], anti-rabies immune globulin (HyperRAB®, Grifols) [24] and anti-hepatitis B immune globulin (HyperHEP B®, Grifols).

Materials and methods

Manufacturing process

TIG produced by a caprylate/chromatography process (TIG-C) begins with the donation of plasma using plasmapheresis by vaccinated individuals with sufficient titres of anti-tetanus antibodies. Donors undergo rigorous screening for many common blood-borne diseases and following current regulatory and industry guidelines [9–11].

The pooled plasma from vaccinated donors was subjected to alcohol fractionation and then treatment with caprylate and purification by chromatography as previously described for Gamunex®-C (IGIV-C), a preparation of human immune globulin [18] and other hyperimmune globulins [22–24]. The hyperimmune globulins differ in that the final protein concentration is 15–18% compared to 10% for IGIV-C. All of these products are subjected to steps in the caprylate/chromatography process that have been demonstrated to eliminate blood-borne viruses in the unlikely event that any should be present [25]. The steps in the caprylate/chromatography process are shown in Figure 1 along with the steps in the solvent/detergent process (S/D). Immunoglobulin products produced by the previously used S/D process were used as comparators to TIG-C.

Product characterisation

All product characterisation methods were cross-validated to confirm suitable performance and equivalency with all the product matrices analysed.

Formulation properties

As part of the delineation of the characteristics of TIG-C (HyperTET®, Grifols), manufacturing standards were created, and comparisons were made with immunoglobulin (IG) products manufactured using the S/D process (HyperHEP B® S/D, HyperRAB® S/D, GamaSTAN® S/D; Grifols) as shown in Table 1. Data from this group of products made by the S/D process were combined and is referred to as IGIM-S/D. All these S/D products are manufactured using the same processes and differ only in the immunisation status of the plasma donors, i.e. donors

Table 1. Characteristics of tetanus immune globulin-caprylate/chromatography process (TIG-C)

	TIG-C	TIG-S/D	IGIV-C, 10%
Nominal anti-tetanus Potency (units[a])	250	250	NA
Protein (%)	15–18	15–18	9–11
pH	4.1–4.8	6.4–7.2	4.0–4.5
Glycine (M)	0.16–0.26	0.21–0.32	0.16–0.24
Chloride, as NaCl%	NA	0.4–0.5	NA
Route of administration	IM	IM	IV and SC
Package sizes	250 Units	250 Units	1, 2.5, 5, 10, 20, 40 g

Tetanus immune globulin manufactured by the caprylate/chromatography process (TIG-C), compared to TIG manufactured by the solvent/detergent process (TIG-S/D), and intravenous immunoglobulin manufactured by the caprylate/chromatography process (IVIG-C).
[a]Antitoxin units (AU)/mL.

Table 2. IgG purity and molecular distribution in tetanus immune globulin – caprylate/chromatography process (TIG-C)

	TIG-C, 16.5% ($n = 4$)	IGIM-S/D, 16.5% ($n = 6$)	IGIV-C, 10% ($n = 360$)
Purity (% IgG)	99.0 ± 0.3	100.0 ± 0.0	100.0 ± 0.0
Molecular size distribution			
Monomers plus dimers (%)	99.8 ± 0.5	99.0 ± 0.0	100.0 ± 0.0
Polymers + aggregates	⩽1	1.0 ± 0.0	<1
Fragments (%)	<1	<1	<1
IgA (μg/ml)	140 ± 70	180 ± 23	52 ± 16
IgM (μg/ml)	<50	150 ± 38	<5
Albumin (μg/ml)	<10	80 ± 37	<2

Tetanus immune globulin produced by the caprylate/chromatography process (TIG-C), immune globulins for intramuscular administration made by the solvent detergent process (IGIM-S/D) and IG for intravenous administration made by the caprylate/chromatography process (IGIV-C). Values are the mean ± s.d. or less than the limits of quantitation (<) for the respective assay. Assays have different sensitivities based on protein concentration and amount of dilution required. n = the number of batches of product tested.

for the hepatitis B product are vaccinated against hepatitis B, and donors for the rabies product are vaccinated against rabies.

Molecular characteristics

Size exclusion chromatography (as previously described [19]) was used to quantify the molecular forms of IgG in the TIG-C formulation. The area of each peak (monomers, dimers, aggregates and fragments) was expressed as a percentage of the total area of the combined peaks.

Purity

Capillary zone electrophoresis (Beckman Coulter, Fullerton, CA, USA) and agarose membrane electrophoresis (Sebia, Lisses, France) were used to measure the purity of TIG-C. These two methods have been internally validated to produce comparable results. The content of IgG and select residual proteins (IgA, IgM and albumin) were measured by immunonephelometry (BNII nephelometric system, Siemens Healthcare Diagnostics, Inc., Tarrytown, NY, USA).

The presence of residual pro-coagulant factors in TIG-C was also measured. Enzyme-linked immunosorbent assays (ELISA) were used to measure Factor XI content (Molecular Innovations, Inc., Novi, MI, USA), using the World Health Organization (WHO) 2nd International Standard for Factor XI (Code 15/180) from National Institute for Biological Standards and Control (NIBSC, Hertfordshire, England, UK). Factor XII content was measured using an ELISA from Assaypro, Inc., St. Charles, MO, USA using a FXII standard provided by the manufacturer. Factor XIa activity was tested using the Rossix Rox Factor XIa Kit (Rossix AB, Taljegårdsgatan 3B, SE-431 53 Mölndal, Sweden: American distributor: Diapharma Group, Inc., West Chester, OH, USA) using a standard linked to the WHO 1st International Standard for Factor Xia (Code 13/100, NISBC). A spectrophotometric test [21] was used to quantify the amount of residual prekallikrein activator (PKA) utilising a PKA reference preparation (EDQM, Strasbourg, France).

Residual pro-coagulant activity was also quantified with thrombin generation time (TGT: Technothrombin TGA Kit, Technoclone GmbH, Vienna, Austria: standard Code 13/100, NIBSC) and non-activated partial thromboplastin time (NAPTT: Siemens BCS-XP Coagulation Analyzer, Siemens Healthineers, Tarrytown, NY, USA) tests. The NAPTT was based on the assay described in the European Pharmacopeia [26].

TIG-C potency and specific activity

The potency of TIG-C (antitoxin units (AU)/ml) was measured using an *in vivo* assay in guinea pigs [27]. Dilutions of TIG-C or intermediates from the manufacturing process (1:10, 1:100 and 1:1000) were tested in groups of three guinea pigs per dilution. The diluted TIG-C or intermediate was mixed with US Standard Tetanus Toxin (Center for Biologics Evaluation and Research (CBER), US Food and Drug Administration, Silver Spring, MD, USA) and tested in comparison to US Standard Tetanus Antitoxin (CBER). The animals were observed for up to 144 h after subcutaneous injection of toxin alone (control) toxin plus antitoxin (standard) or toxin plus test material. The potency of the test product was recorded as the dilution at which all three guinea pigs survived longer than the average time for the control group. For a valid test, time of death for the control group must be between 60 and 120 h (historic mean ~96 h).

Specific activity was also calculated at the steps in the manufacturing process when tetanus potency was measured. Specific activity (AU/mg IgG) was calculated by dividing tetanus potency (AU/ml) by IgG concentration (mg/ml).

Table 3. Concentration and activity of coagulation factors in tetanus immunoglobulin – caprylate/chromatography process (TIG-C)

Product	Factor XI (ng/ml)	Factor XIa activity (mIU/ml)	Factor XII (ng/ml)	NAPTT (sec)	TGT (mIU/ml)	PKA Activity (IU/ml)
TIG-C ($n = 4$)	<2.0	⩽1.0	<75	286 ± 37	<0.63	<3.0
IGIM-S/D ($n = 6$)	56 ± 27	51 ± 41	<75	98 ± 15	309 ± 173	58 ± 39
IGIV-C ($n = 4$)	<2.0	<1.0	<75	255 ± 8	<0.63	<1.0

NAPTT, non-activated partial thromboplastin time; TGT, thrombin generation time; PKA, prekallikrein activator. n = the number of batches of product tested.
Tetanus immunoglobulin manufactured by the caprylate/chromatography process (TIG-C), immune globulins for intramuscular administration made by the solvent detergent process (IGIM-S/D) and IG for intravenous administration made by the caprylate/chromatography process (IGIV-C).

Table 4. Subclass distribution of IgG forms for tetanus immunoglobulin – caprylate chromatography process (TIG-C)

Product	IgG 1 (%)	IgG 2 (%)	IgG 3 (%)	IgG 4 (%)
TIG-C ($n = 4$)	63 ± 1	31 ± 1	3.0 ± 0.3	2.8 ± 0.1
IGIM-S/D ($n = 6$)	63 ± 2	27 ± 1	7.7 ± 0.5	2.4 ± 0.7
IGIV-C ($n = 20$)	63 ± 2	30 ± 2	4.2 ± 0.8	3.2 ± 0.4
Normal adult [29]	56 (31–89)	33 (13–63)	7.1 (2.7–13.9)	3.9 (0.6–13.9)

Tetanus immunoglobulin made using the caprylate chromatography process (TIG-C), immune globulins for intramuscular administration made by the solvent detergent process (IGIM-S/D) and IG for intravenous administration made by the caprylate/chromatography process (IGIV-C). n = the number of batches of product tested.

Results

Formulation of final product

The process used to manufacture TIG-C is a modification of the procedures developed for the manufacture of IGIV-C 10% [18, 19]. The TIG-C manufacturing process was modified to produce a higher protein concentration in the final product. The final protein concentration (15–18%) and the anti-tetanus potency (250 Units) for TIG-C were set to be the same as TIG-S/D (Table 1). TIG products are standardised against the US Standard Antitoxin and the US Control Tetanus Toxin and contain not less than 250 tetanus antitoxin units per container [11]. The final pH values for TIG-C and IGIV-C are lower than the value for TIG-S/D but the glycine contents were similar. In contrast to the final formulation of TIG-S/D, TIG-C does not contain significant amounts of NaCl. The TIG-C formulation was based on experience with the IGIV-C product, a lower pH and no added NaCl.

IgG purity and composition

All three of the formulations tested (TIG-C, IGIM-S/D and IGIV-C) were close to 100% IgG purity (Table 2). Small amounts of IgA were detected in all three formulations. The concentration of IgA present in TIG-C was comparable to that seen in IGIM-S/D but higher than that found in IGIV-C 10%. The concentration of IgM in these products was above the limit of quantitation only in IGIM-S/D.

Residual coagulation factors were tested in the final TIG formulation. Table 3 shows the amounts of these residual factors measured in the TIG-C formulation compared to IGs produced using the S/D process. These data show a substantial reduction in coagulation factor impurities by the caprylate/chromatography process. All the coagulation factor impurities analysed were reduced except for Factor XII which was already below quantifiable limits in products generated by the caprylate/chromatography and solvent/detergent processes.

The NAPTT were measured for TIG-C and IGIM-S/D. NAPTT is a global assay for coagulation activity in human plasma. The NAPTT assays gave a result within the same range as the negative control (⩾200 s) [28] for TIG-C compared to below the negative control range for IGIM-S/D. A value below the negative control indicates the presence of pro-coagulant impurities. TGT were also performed to assess the presence of activated coagulation factors. These tests gave values below the limits of detection for TIG-C, but indicated the presence of coagulation factor impurities in IGIM-S/D.

In addition, size exclusion chromatography was used to quantify the distribution of the different molecular forms of IgG: monomers, dimers, aggregates and fragments. TIG-C and IGIV-C had close to 100% of the monomeric and dimeric forms of IgG. The polymeric forms of IgG were detectable only in the IGIM-S/D products (at the lower limit of quantitation) and fragments were below quantitation limits in all products.

Relative contribution of IgG subclasses

The contribution of IgG subclasses to the total IgG content was measured for TIG-C and compared to IGIM-S/D, IGIV-C and the normal adult range (Table 4). The subclass distribution for the three products vary but within the range of expected values [29]. For all three products, the mean IgG1 percentage was slightly higher than the normal adult mean value and the IgG2 and IgG4 were slightly lower. For TIG-C and IGIV-C, the mean values for percentage of IgG3 were lower than the normal adult mean value and for IGIM-S/D the mean IgG3 percentage was slightly higher.

Changes in potency and specific activity during manufacture of TIG-C

The anti-tetanus potency (in AU/ml) and specific activity (in AU/mg IgG) of TIG-C were measured in the pooled plasma, master bulk and final product stages. As shown in Figure 2, anti-tetanus potency increased from the plasma pool (16 ± 3 AU/ml) to the

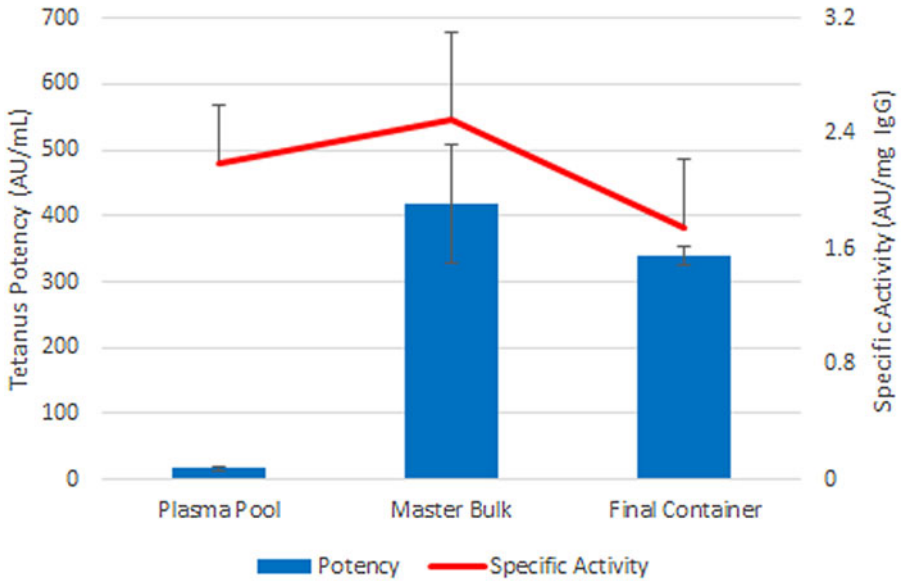

Fig. 2. Tetanus potency and specific activity of tetanus immune globulin during manufacture by the caprylate/chromatography process (TIG-C). Data are expressed as the mean ± s.d. A marginal dilution may occur from Master Bulk to Final container to target potency/ml of product.

master bulk stage (419 ± 90 AU/ml) and was diluted into the final product (338 ± 14 AU/ml). In contrast the specific activity, a measure of the anti-tetanus activity per mg of IgG, remained relatively constant across the manufacturing process. Specific activity in the plasma pool was 2.2 ± 0.4 AU/mg IgG and increased slightly to 2.5 ± 0.6 AU/mg IgG then decreased to 1.8 ± 0.5 AU/ml in the final product.

Discussion

Vaccinated plasma donors undergo health screening and their donated plasma units are subject to rigorous testing to be sure they are free of infectious agents [30]. There were no changes in the immunisation of plasma donors for TIG-C *vs.* TIG-S/D purification processes; the starting plasma remains the same for both processes.

In the event that infectious agents are present, the caprylate/chromatography process for purification of immunoglobulins incorporates several steps to remove and/or inactivate blood-borne viruses. During the manufacturing process, caprylate precipitates impurities and infectious agents that are then separated from the product by depth filtration [31]. Additional incubation with caprylate has been shown to disrupt the lipid envelope and thereby inactivating any lipid-encased virions present [32, 33]. Additional clearance of any residual infectious agents occurs in this manufacturing process during the chromatography, filtration and low pH incubation steps [34].

In addition to the removal/inactivation of potential blood-borne pathogens, the caprylate/chromatography process is effective at removing coagulation factor impurities from the final product [20]. The Factor XI content was reduced to below detection level in the final product. This reflects a removal of >12 500 fold from the Fraction II + II Paste suspension step. Similarly, Factor XII content was also reduced during manufacturing (>620-fold) to below detection in the final product. In contrast, the S/D process resulted detectable levels of Factor XI. The S/D process also shows coagulation activity in the Factor XIa, NAPTT, TGT and PKA results.

The caprylate/chromatography process described for TIG-C was based on the established methods for manufacturing 20% IGSC (Xembify®, Grifols, Clayton, NC, USA) and 10% IGIV (Gamunex-C®, Grifols) [19, 20]. In addition, this process is being utilised for the production of concentrated immune globulins for other diseases, e.g. COVID-19 [22], rabies [24] and Ebola [23]. Based on the clinical trial data generated during the development of these products and the post-marketing surveillance, the caprylate/chromatography process yields safe and effective immune globulin products.

As shown in Figure 2, anti-tetanus potency increased approximately 25-fold from pooled plasma to TIG-C in the final container. In contrast, comparison of the specific activity across the manufacturing process shows that it remained relatively constant. This reflects the concentration of all IgG throughout the process and not a selective increase in anti-tetanus IgG. However, the increase in anti-tetanus potency through the caprylate/chromatography process allows the administration of a more concentrated product and a smaller volume compared to the original plasma pool. In addition, the consistency of specific activity from the plasma pool to final product indicates that the processing conditions have not reduced the activity of the starting IgG. The decrease in specific activity from the bulk to the final product is due to dilution with normal immunoglobulin, which is added to maintain a standardised potency for dosing.

In conclusion, as described above, there are many procedures (rigorous testing, health screening and manufacturing processes) designed to protect the integrity of the plasma supply and plasma-derived products (e.g. TIG-C and other immune globulins) from blood-borne pathogens. At the same time, coagulation factor impurities, present in plasma, are removed in the caprylate/chromatography process. These processes have taken TIG, a product with a long history of safe and effective use, and made it purer.

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Conflict of interest. All authors on this paper are/were employees of Grifols at the time the work was performed.

Data availability statement. The data underlying these studies are available from the corresponding author upon reasonable request.

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