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# Batch-to-batch reproducibility of Transferon™

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ABSTRACT

Human dialyzable leukocyte extracts (DLEs) are heterogeneous mixtures of low-molecular-weight peptides that modulate immune responses in various diseases. Due their complexity, standardized methods to identify their physicochemical properties and determine that production batches are biologically active must be established. We aimed to develop and validate a size exclusion ultra performance chromatographic (SE-UPLC) method to characterize Transferon™, a DLE that is produced under good manufacturing practices (GMPs). We analyzed an internal human DLE standard and 10 representative batches of Transferon™, all of which had a chromatographic profile characterized by 8 main peaks and a molecular weight range between 17.0 and 0.2 kDa. There was high homogeneity between batches with regard to retention times and area percentages, varying by less than 0.2% and 30%, respectively, and the control chart was within 3 standard deviations. To analyze the biological activity of the batches, we studied the ability of Transferon™ to stimulate IFN-γ production *in vitro*. Transferon™ consistently induced IFN-γ production in Jurkat cells, demonstrating that this method can be included as a quality control step in releasing Transferon™ batches. Because all analyzed batches complied with the quality attributes that were evaluated, we conclude that the DLE Transferon™ is produced with high homogeneity.



## 1. Introduction

Human dialyzable leukocyte extracts (DLEs) are heterogeneous mixtures of low-molecular-weight peptides (<10 kDa) that are released on disruption of peripheral blood leukocytes from healthy donors [1]. Administration of DLEs improves clinical responses in infections [2,3], allergies [4], cancer [5,6], and immunodeficiencies [7].

The therapeutic and adjuvant effects of DLEs are associated with their ability to modulate immune responses in the above mentioned diseases (for detailed reviews see Refs. [8,9]). DLEs alter innate signaling pathways, such as TLRs, NF-κB, and cAMP in cultured immune cells [10]. Consequently, DLEs stimulate the production of proinflammatory cytokines, including TNF-α, IL-6 [11], and IFN-γ [12].

Despite the positive clinical effects of DLEs, their complexity has impeded full characterization of their components and active substances. Thus, characterization, total protein per dose, and efficacy of DLEs have never been reported, necessitating the development and validation of suitable methods that analyze their physicochemical properties. In addition, such methods would allow

*Abbreviations:* IFN-γ, interferon gamma; HPLC, high-performance liquid chromatography; UPLC, ultra-performance liquid chromatography; SE, size exclusion; RP, reverse-phase; GMPs, good manufacturing practices; SD, standard deviation; RSD, relative standard deviation; DLE, dialyzable leukocyte extract; Da, daltons; NF-κB, nuclear factor kappa B; cAMP, cyclic adenosine monophosphate; TNF-α, tumor necrosis factor alpha; IL-6, interleukin 6; DTH, delayed-type hypersensitivity; Gly, glycine; Ser, serine; Glu, glutamine; BCA, bicinconinic acid; ICH, International Conference of Harmonization; FDA, Food and Drug Administration.

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establishing the relationship between the physicochemical properties and expected biological activities of DLEs.

The seminal reports on the physicochemical characterization of DLEs have applied chromatographic methods. In 1962, Baram and Mosko used reverse-phase (RP) chromatography to demonstrate that DLEs are a heterogeneous mixture that comprise diverse chromatographic fractions [13]. This group also observed that DLEs generated 10 peaks by size exclusion chromatography—the fifth peak, containing molecules under 10 kDa, harbored most of the biological effects [14,15].

Similarly, Vandenbark and colleagues [16], Burger and colleagues [17], and Khan and colleagues [18] noted that specific subfractions, obtained by high-performance liquid chromatography (HPLC), RP-HPLC, and anion-exchange chromatography, respectively, mediated the effects of DLEs. The active fraction was susceptible to proteases [17], and its physicochemical properties suggested high protein content [18]. On further analysis of the active fractions of the DLEs, the most abundant amino acids were Gly, Ser, and Glu, whereas Lys and aromatic amino acids were the least abundant [19].

Kirkpatrick and colleagues provided definitive evidence that the peptidic content mediates the biological activity in DLEs. They fractionated DLEs by RP-HPLC and isolated peptides that contained the sequence MXLLYAQDL/VEDN. Administration of blocking peptides inhibited the characteristic transfer of delayed type hypersensitivity (DTH) by DLEs [20]. Collectively, these reports demonstrate that DLEs are a heterogeneous mixture of polar and hydrophilic peptides with molecular weights below 10 kDa.

DLEs are produced and commercialized worldwide. Transferon™ is a DLE manufactured by the National School of Biological Sciences (ENCB), National Polytechnic Institute (IPN), Mexico, at GMP facilities. Transferon™ is registered by Mexican health authorities as a drug and commercialized nationally. Similarly, other DLEs are registered as drugs in China, Cuba, and Czech Republic [21–23]. Based on the significance of chromatographic methods in developing and characterizing DLEs and the lack of a standard physicochemical method to analyze them, we aimed to develop and validate an UPLC approach to examine Transferon™. We identified a typical chromatographic profile with eight main peaks.

We also assessed the batch-to-batch reproducibility of 10 representative batches of Transferon™ by analyzing their physicochemical properties and their ability to effect IFN-γ secretion *in vitro*. All batches met the quality control standard with regard to peptide concentration, molecular weight rate, time of retention of main peaks, and efficacy.

## 2. Materials and methods

### 2.1. Analytical samples and peptide quantification

Samples of Transferon™ were obtained from 10 typical batches (10K02B, 10K04A, 11A01E, 11A01C, 11A02D, 11A02E, 11A02F, 11A02G, 11E01, and 12A01) produced by UDIMEB at GMP facilities using a modified version of the method of Borkowsky and Lawrence [24]. Briefly, leukocytes were lysed with 5 freeze-thaw cycles, and dialysis was performed using a 12-kDa membrane to obtain low-molecular-weight peptides in the permeate. The peptide concentration was measured by bicinchoninic acid (BCA) method using the Pierce BCA kit (Thermo Fisher Scientific; Massachusetts, USA) according to the manufacturer's instructions [25]. Internal batch pattern of Transferon™ was defined as an internal batch that has been approved satisfactorily all the quality control tests specified for the product (endotoxin content, microbiologic test, characterization, protein content and biological activity).

### 2.2. Chromatographic methods

We performed SE-UPLC on an H class Acquity™ UPLC system (Waters™; Massachusetts, USA) that was controlled by Empower™ (Waters™) using an Acquity BEH 125 molecular size exclusion column (1.7 μm × 4.6 × 150 mm) (Waters™) at 30 °C. The chromatograms were generated on a tunable ultraviolet (TUV) detector (Waters™) at 280 nm with an isocratic workflow of 0.4 ml/min of monobasic/dibasic sodium phosphate-buffered solution (pH 6.8, 50 mM; Mallinckdrodt Baker; Pennsylvania, USA), sodium chloride 150 mM (Mallinckdrodt Baker) and ultrapure Milli Q water (Millipore, Darmstadt, Germany). The run time was 15 min.

### 2.3. Characterization analysis: SE-UPLC

SE-UPLC is considered as a characterization method per the International Conference of Harmonization (ICH) [26]. The method was validated by testing system suitability, precision, and specificity. We used a gel filtration standard (Bio-Rad™; California, USA) spiked with L-tryptophan BioUltra (Sigma Aldrich; Missouri, USA) as molecular weight standard. To analyze the batch-to-batch reproducibility, Transferon™ samples were compared against an internal batch pattern (1.5 mg/ml); each sample was injected in duplicate (1 μl for standards and 3 μl for Transferon™ samples). The chromatographic profiles were analyzed using Empower™ (ApexTrack method) to obtain the area percentage and retention time for each peak.

### 2.4. Biological activity

The efficacy of Transferon™ was determined in Jurkat clone E6-1 cells (ATCC; Manassas, VA, USA). Briefly, $3 \times 10^5$ cells were plated in 24-well plates and maintained in RPMI 1640 medium (ATCC; Virginia, USA) that was supplemented with fetal bovine serum (Gibco™, Life Technologies; New York, USA). Cultures were stimulated with Transferon™ (0.1, 1, or 10 μg/ml) or type IV-S concanavalin A (25 μg/ml; Sigma–Aldrich; Missouri, USA) as a positive control. After 72 h, IFN-γ was measured in the supernatant using the BDOptEIA-IFNγ ELISA kit (Becton Dickinson Biosciences; California, USA) by triplicate. After stimulation, 100 μl of standard or sample were mixed with 50 μl of PBS with 10% of heat inactivated FBS and incubated for two hours in capture antibody-coated wells. After extensive washing, 100 μl of a solution including a biotinylated detection antibody and streptavidin-horseradish peroxidase were added to each well and incubated for one hour. After washing, the reactions were developed with 3,3′5,5′-Tetramethylbenzidine (TMB) plus hydrogen peroxide, stopped, and read immediately at 450 nm [27]. IFN-γ quantification above the detection limit (4.7 pg/ml) was considered as positive, since basal production of this cytokine in Jurkat cells was undetectable.

### 2.5. Data analysis

To analyze the homogeneity in chromatographic profiles between Transferon™ batches, we calculated the average, standard deviation, and percentage standard deviation for the peak areas and retention times. We also calculated the average and standard deviation for peptide content and IFN-γ.

## 3. Results and discussion

DLEs and their clinical activity have been described since 1954 [28], but their batch-to-batch reproducibility in GMP facilities has not been examined. We analyzed 10 batches of Transferon™ with

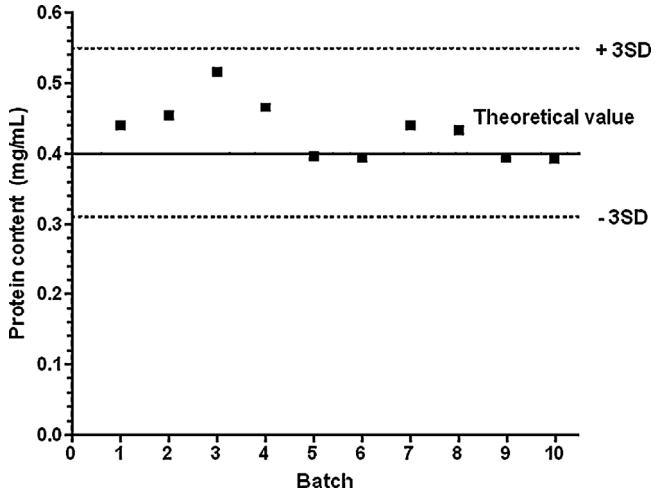


**Fig. 1.** Analysis of protein content between Transferon™ batches. Protein concentration, quantified by BCA method, is specified for each batch (squares). Theorical value (solid line) and ±3SD interval (dashed lines) are shown.

**Table 1**
Validation parameters for the SE-UPLC identity method developed for Transferon™.

| Parameter | Acceptance criteria | Result |
|---|---|---|
| System suitability | Tailing factor less than 1.75 | Tailing factor 1.44–1.51 |
| | Relative retention time (against B12 vitamin) of: | Relative retention time: |
| | Mioglobulin 0.67 ± 0.05 SD | Mioglobin 0.67 ± 0.00 |
| | Ovoalbumin 0.56 ± 0.05 SD | Ovoalbumin 0.57 ± 0.00 |
| Method accuracy | Variation of areas between batches lectures are less than 3.0% | %RSD 0.45–1.76 |
| System accuracy | Variation of areas between three standard lectures are less than 3.0% | %RSD 1.15–2.28 |
| Specificity | Correspondence between internal batch pattern and batches of Transferon™ | Chromatographic profiles of batches are equal to internal batch pattern standard |

regard to protein content, characterization by SE chromatography-UPLC, and activity in an *in vitro* model of IFN-γ production. The batches were typical production batches from 2010 and 2011.

Early reports obtained DLEs on a low scale using cells from patient-related or DTH-positive donors [13]. In these studies, dose was calculated in arbitrary units that were related to the number of leukocytes of origin [29]. In contrast, each Transferon™ batch is produced from a pool of 1000 healthy donors in order to reduce donor-to-donor variability [30]. Further, the active principle per dose in Transferon™ is quantified as protein content, as reported [31,32]. Transferon™ contains 2 mg of peptides in 5 ml of excipient. By analysis of protein content in 10 batches, Transferon™ had an average content of 0.433 mg/ml, which represent a 108.2% regarding to the theoretical expected value of 0.40 mg/ml. However, this percentage meets the criteria established in the specification for Transferon™, which is from 0.34 to 0.46 mg/ml. The variation in concentrations between batches was within ±3 SD (Fig. 1), demonstrating that the process by which Transferon™ is produced consistently generates batches that are homogeneous with regard to protein content.

### 3.1. SE-UPLC characterization method

According to the FDA's Guide for Biological Products and Mexican Pharmacopeia, biological drugs for clinical use require characterization of the active principle during production and as part of the final quality control step [33,34]. Since Transferon™ is currently as project to be included in the Mexican Pharmacopeia, we used chromatography, which is a general method for drug characterization [33,34]. We developed a characterization method for Transferon™ using SE-UPLC. We choose to use columns with a 125 Å pore size particles (suitable for low molecular weight peptides separation) and tested two column lengths: 150 and 300 mm. The resolution factor ($\alpha$) for peaks 3 and 4 was 1.02 and 1.08 respectively for 150 mm column, and 1.45 and 1.50 for 300 mm column. Despite of the better resolution in the 300 mm column, peaks 1, 3 and 4 were not base resolved. Because of that, size exclusion column of 150 mm was established for routine release analysis in quality control laboratory.

To validate the assay, we analyzed its suitability, accuracy, and specificity (Table 1). The system's suitability was examined by comparing the retention times for molecules with known molecular weights (standards) and calculating the tailing factor. To study the accuracy of the method and system, we calculated the variations in peak areas between batches and between 3 standard lectures. In both cases the variation was less than 3 SD. Specificity was evaluated by comparing samples with our internal standard of Transferon™. The chromatographic profiles of the batches were the same as that of the internal batch pattern. These data demonstrate that our characterization assay meets the minimum requirements for qualitative physicochemical methods, as indicated by the International Conference on Harmonization [26].

With this validated method, we analyzed 10 typical batches of Transferon™ at the moment of release. Because DLEs are a complex mixture of peptides, they generate multiple chromatographic peaks. Other studies have reported 4, 7, and 13 peaks for DLEs that have been produced by similar processes [35–39]. In our assay, Transferon™ generated 8 main peaks, as did the internal batch pattern. The difference between our data and the earlier reports can be explained by disparities in the sensitivity of the chromatographic methods [40].

All peaks of Transferon™ were shown in the range of 17 kDa (equine myoglobine) to 0.2 kDa (tryptophan) (Fig. 2A). The fifth peak had the largest area (28.09%), while the seventh peak had the minor area (0.77%). These properties are consistent with a product that has been dialyzed against a 12 kDa membrane, such as DLEs [24].

Next, we compared the area percentages and retention times of the 8 main peaks to analyze batch-to-batch reproducibility. The %RSD for all area percentages was below 30% (Table 2) and varied within 3 SD (data not shown). This variation is accepted as a

**Table 2**
Comparison of area percentages of the eight main peaks in Transferon™ chromatographic profile between batches.

| | BATCH | P1 | P2 | P3 | P4 | P5 | P6 | P7 | P8 |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 10K02B | 4.2 | 14.75 | 3.45 | 17.55 | 23.1 | 13.7 | 1.35 | 21.4 |
| 2 | 10K04A | 5.4 | 24.55 | 2.85 | 14.3 | 24.15 | 9 | 0.9 | 17.7 |
| 3 | 11A01B | 8.85 | 26.15 | 2.5 | 11.95 | 24.9 | 10.8 | 0.75 | 13.6 |
| 4 | 11A01C | 8.85 | 14.65 | 3.3 | 15.05 | 24.2 | 12.35 | 0.7 | 20.5 |
| 5 | 11A02D | 5.7 | 22.5 | 2.95 | 12.45 | 27.4 | 9.9 | 0.6 | 17.45 |
| 6 | 11A02E | 5.45 | 23.35 | 2.3 | 11.2 | 31.15 | 10.25 | 0.75 | 14.35 |
| 7 | 11A02F | 6.3 | 25.65 | 2.7 | 10.05 | 29.4 | 10.25 | 0.7 | 13.75 |
| 8 | 11A02G | 5.35 | 23.7 | 2.3 | 10.85 | 32.05 | 10.35 | 0.7 | 13.7 |
| 9 | 11E01 | 3.4 | 20.2 | 1.55 | 10.45 | 35.3 | 15.55 | 0.65 | 11.45 |
| 10 | 12A01 | 7 | 28.35 | 1.35 | 12.1 | 29.2 | 8.6 | 0.6 | 10.65 |
| MEAN | | 6.05 | 22.39 | 2.53 | 12.6 | 28.09 | 11.08 | 0.77 | 15.46 |
| %RSD | | 29.41 | 20.56 | 27.07 | 18.76 | 14.35 | 19.58 | 28.75 | 23.59 |

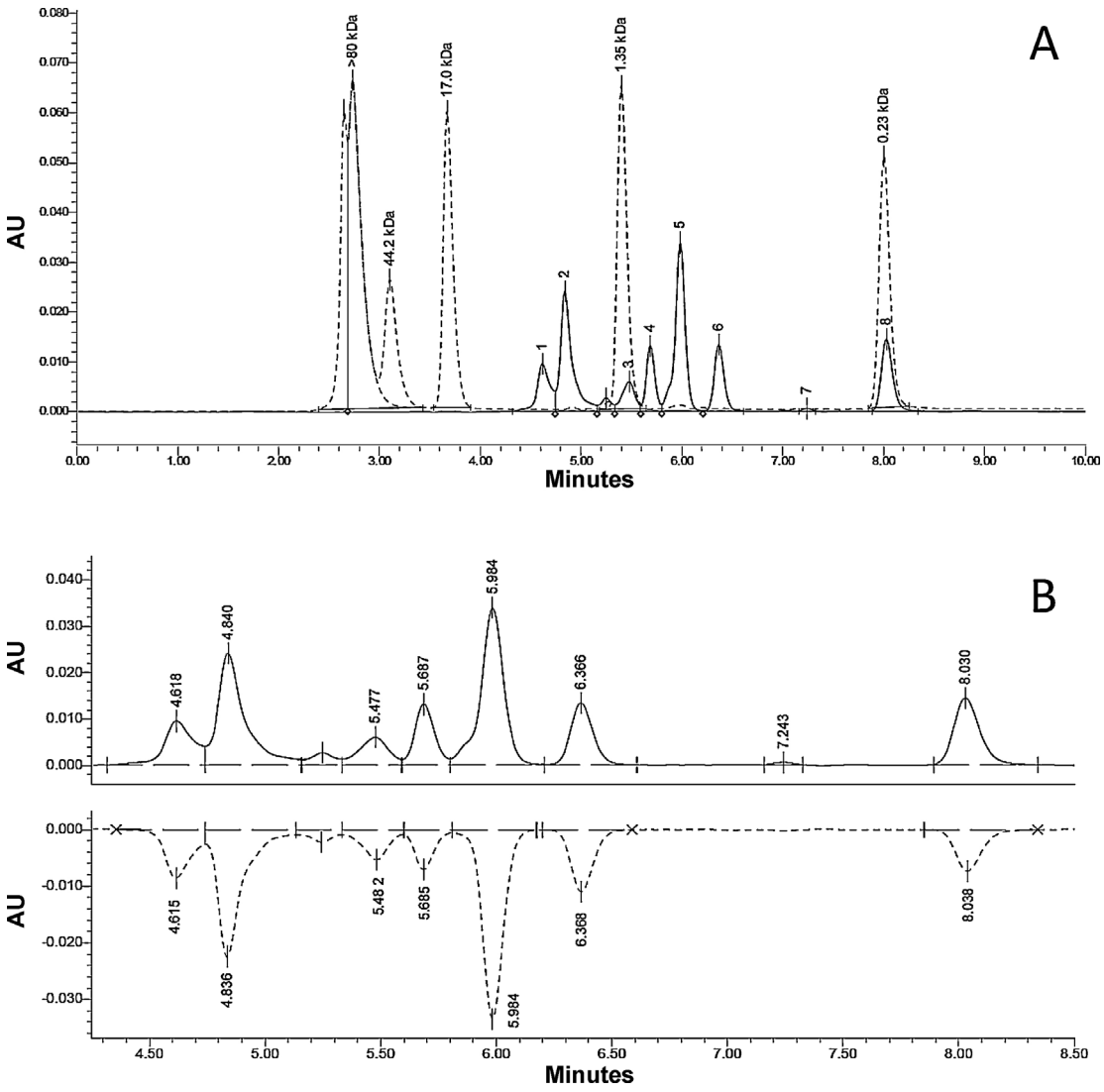


**Fig. 2.** SE-UPLC chromatographic profile of Transferon™. A. Comparison between a representative batch of Transferon™ (solid line) and molecular weight standards (dashed line), showing that the eight main peaks of Transferon™ have retention times between those of equine myoglobin (17.0 kDa) and tryptophan (0.23 kDa). Other peaks in the standard correspond to tryglobulin/gamma-globulin (>80 kDa), ovoalbumin (44.2 kDa), and B12 vitamin (1.35 kDa). The chromatographic profiles were analyzed using Empower™ (ApexTrack method) to obtain the area percentage and retention time for each peak. B. Representative identity test for Transferon™ (above) compared against an internal human DLE standard (below). All batches showed homogeneity in the test. AUs: Absorbance Units.

quality specification of a biological product by regulatory agencies and allows the product to have a biological activity. For example, the European Pharmacopeial specification of erythropoietin isoform 6 has a range from 10% to 35% area, showing 50% variation [41]. Similarly, the variation in retention times for each peak from batch to batch was below 0.2% (Table 3) and varied within 3 SD (data not shown). Area percentages and retention times depend on the physicochemical attributes of a product; thus, our results indicate that the homogeneity between Transferon™ batches can be tracked for at least 2 years, complying with the minimum quality control standards for the pharmaceutical industry [42].

### 3.2. Biological activity

Efficacy is an essential attribute of a therapeutic drug, and its activity is expected to be preserved between commercial batches [33]. DLEs have demonstrated activity in clinical trials from the past 50 years [2,8,43–45], but only recently they have begun to be produced by GMPs, necessitating the development of tests that evaluate their activity and batch release.

To this end, animal models and *in vitro* assays are preferred for ethical, timing, and commercial reasons. For example, the activity of DLEs has been demonstrated by inducing DTH in mice [46] or analyzing their effects on leukocyte migration [47]. Although

**Table 3**
Comparison of retention times of the eight main peaks in Transferon™ chromatographic profile between batches.

| | BATCH | P1 | P2 | P3 | P4 | P5 | P6 | P7 | P8 |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 10K02B | 4.7 | 4.9 | 5.49 | 5.76 | 5.94 | 6.42 | 7.19 | 8.04 |
| 2 | 10K04A | 4.68 | 4.9 | 5.5 | 5.76 | 5.94 | 6.43 | 7.2 | 8.05 |
| 3 | 11A01B | 4.66 | 4.89 | 5.49 | 5.76 | 5.95 | 6.43 | 7.21 | 8.07 |
| 4 | 11A01C | 4.66 | 4.9 | 5.49 | 5.76 | 5.95 | 6.43 | 7.21 | 8.06 |
| 5 | 11A02D | 4.68 | 4.9 | 5.49 | 5.75 | 5.93 | 6.42 | 7.21 | 8.06 |
| 6 | 11A02E | 4.68 | 4.9 | 5.49 | 5.75 | 5.94 | 6.43 | 7.21 | 8.06 |
| 7 | 11A02F | 4.68 | 4.9 | 5.48 | 5.75 | 5.94 | 6.42 | 7.21 | 8.06 |
| 8 | 11A02G | 4.68 | 4.9 | 5.49 | 5.75 | 5.94 | 6.42 | 7.22 | 8.06 |
| 9 | 11E01 | 4.7 | 4.9 | 5.48 | 5.75 | 5.94 | 6.43 | 7.22 | 8.07 |
| 10 | 12A01 | 4.67 | 4.89 | 5.49 | 5.76 | 5.94 | 6.42 | 7.2 | 8.05 |
| | MEAN | 4.68 | 4.90 | 5.49 | 5.76 | 5.94 | 6.43 | 7.21 | 8.06 |
| | %RSD | 0.21 | 0.08 | 0.11 | 0.17 | 0.07 | 0.07 | 0.13 | 0.11 |

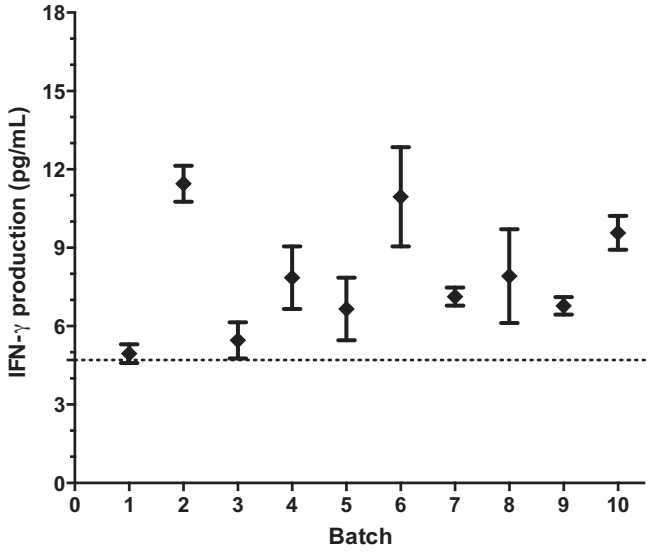


**Fig. 3.** Effect of Transferon™ on IFN-γ secretion by Jurkat cells. Cells were treated for 72 h with different Transferon™ batches, and the concentration of IFN-γ in supernatants was quantified by ELISA (diamonds). Each experiment included positive (25 μg/ml concanavalin A) and negative (vehicle) controls (not shown). All batches induced IFN-γ secretion above the method's detection limit (dashed line). Data are shown as mean ± SEM (triplicates).

animal models reflect the complexity of a drug response in a whole organism, we developed an *in vitro* assay as a quality control for the release of Transferon™ batches to avoid the variability between animal models, minimize the number of animals that is employed during Transferon™ production, and reduce the analysis time.

DLEs upregulate IFN-γ in lymphocytes [48], and our assay analyzed the ability of a lymphocytic cell line (Jurkat clone E6-1) to produce IFN-γ in response to Transferon™. Our positive and negative controls were concanavalin A- and vehicle-treated cells, respectively. All batches induced IFN-γ production to levels above the detection limit (4.7 pg/ml; Fig. 3) and within 3 SD (data not shown). These results contrast previous attempts to develop *in vitro* assays to evaluate DLE activity, such as leukocyte proliferation [49].

Thus, all analyzed batches shared physicochemical properties and consistently generated a biological response—i.e., IFN-γ production.

## 4. Conclusions

Our analysis of 10 batches of Transferon™, produced in GMP facilities, demonstrates that is possible to produce a mixture of peptides that have been extracted from complex raw materials, such as lysed human leukocytes, with high batch-to-batch reproducibility. The 10 batches complied with quality standards for their attributes, such as peptide concentration in the final dose, molecular weight, time of retention of the main peaks, and activity. Thus, Transferon™ is a DLE that observe the government normativity required for hemoderivatives used for clinical use, including batch-to-batch reproducibility.

## Authors' contributions

SV-L, GP-S, and NS-J performed the experiments. GM-R, LP, SE-P, MV-V, and EM-R analyzed the data and wrote the manuscript. SMP-T, MV-V, and EM-R designed the experiments. All authors read and approved the final manuscript.

## Role of funding source

All authors declare that the funding sources had no impact on the study design; the collection, analysis, and interpretation of data; the writing of the report, or the decision to submit the paper for publication.

## Conflict of interest

SV-L, GP-S, NS-J, SE-P and SMP-T are employees or haven compensated for their work at "Proyecto Factor de Transferencia/UDIMEB" the producer of Transferon™. All other authors declare no competing interests.

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