RESEARCH

Viral inactivation capacity of Melagenina® Plus during the storage step

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ABSTRACT

This work was aimed at validating the viral inactivation property during the storage step of the production process of Melagenina® Plus, which is a Cuban biological product for the treatment of vitiligo. The product was challenged at storage with high loads of four viral models, corresponding to three enveloped and one non-enveloped virus, two of them RNA and the other two DNA viruses: the human immunodeficiency virus type 1 (HIV-1), the bovine viral diarrhea virus (BVDV), the porcine herpes virus type 1 (PHV-1) and the canine parvovirus (CPV). The viral titer was determined using the Reed-Muench method based on the viral cytopathic effect, and reduction factors were calculated as the difference of viral loads at the beginning and the end of the step. Enveloped viruses were inactivated between days 1 to 3, and the enveloped virus (CPV) was achieved after 21 days. The viral load showed a very highly significant decrease ($p < 0.0001$), being conditioned to storage temperature (30 ± 5 °C) and ethanol concentration (71 % minimum). The reduction factors achieved on this step (1:5.0 log for HIV-1; 3.5 log for BVDV; 4.24 log for PHV-1 and 5.8 log for CPV) characterized the adequate level of safety of the Melagenina® Plus production process.

Keywords: Melagenina Plus, storage, inactivation, reduction factors

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RESUMEN

Propiedad de inactivación viral durante la obtención de la Melagenina® Plus en la etapa de almacenamiento. Este artículo presenta la validación de la propiedad de inactivación viral de la Melagenina® Plus en la etapa de almacenamiento: producto biológico cubano para el tratamiento del vitiligo. La etapa se retó con altas cargas virales de cuatro modelos virales de tres virus envueltos y uno no envuelto, y de genoma ARN (dos) o ADN (dos), respectivamente: virus de la inmunodeficiencia humana tipo 1 (VIH-1), virus de la diarrea viral bovina (VDVB), virus del herpes porcino tipo 1 (VHP-1) y parvovirus canino (PVC). El título viral se determinó por el método de Reed y Muench basado en el efecto citopático viral; y los factores de reducción se calcularon por la diferencia de la carga viral al inicio y al final de la etapa. La inactivación de los virus envueltos se logró entre el primero y el tercer día, y la inactivación del modelo de virus no envuelto (PVC), a los 21 días. La disminución de la carga viral fue altamente significativa ($p < 0.0001$), determinada por la temperatura de almacenamiento (30 ± 5 °C) y la concentración de alcohol (mínima: 71 %). Los factores de reducción alcanzados en esta etapa para los modelos virales (VIH-1: 5.0 log; VDVB: 3.5 log; VHP-1: 4.24 log; PVC: 5.8 log) le confieren un adecuado nivel de seguridad al proceso de producción de la Melagenina® Plus.

Palabras clave: Melagenina Plus, almacenamiento, inactivación, factor de reducción

Introduction

Human placenta is an organ enriched on active and safe biological substances which are used to produce medicines and cosmetics. One of them is Melagenina® Plus, an internationally reknown pharmaceutical product used for the treatment of vitiligo, which is a dermatological disease affecting 1 % of the world population [1, 2].

Its biological origin forces the product to fulfill both national and international regulatory standards, (in Cuba from the State Center for Medicines Control, CECMED), and also suffice patients' needs.

Those regulations begin with the control during selection and testing of human placenta as raw material. This organ could be naturally infected by a wide range of DNA or RNA viruses [3-5], with an underlying risk for infectious agents transmission.

In order to circumvent limitations inherent to studying placenta (having to evaluate a wide range of viral contaminants, the lag period of viral infections and the lower sensitivity assay levels required), the viral clearance capacity of the given production process needs to be evaluated. This guarantees the pharmaceutical's safety and diminishes chances for any virus from placenta (known or undiscovered, unexpectedly found or dangerous) to remain in the final formulation, being removed or inactivated [6, 7].

In the case of Melagenina® Plus, it is generated by adding calcium to the active pharmaceutical ingredient (API) of Melagenina® lotion. Previously, validation of the Melagenina® lotion manufacturing process demonstrated that this placental extract's safety was conditioned to its storage in ethanol (71 % or higher) and at room temperature (30 ± 5 °C) [8].

Considering that the production processes of these two human placental extracts only differ in the addition of calcium to the API, this work was aimed at

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verifying to what extent this change would impact the safety of the Melagenina® Plus regarding viral inactivation during the storage step.

Materials and methods

This research was performed in the AIDS Research laboratory (Lisida, Cuba), equipped with the necessary resources for the inverse scaling of the process and its validation. There were the proper qualified personnel from the Placenta Derivatives Plant (Deplacén, Cuba), and procedures complied with the standards recommended by the national and international regulatory agencies [3, 7, 9].

Sampling

Two lots of Melagenina® Plus were used in the study and one with the Melagenina® lotion as control. Class A ethanol (95 %) and calcium chloride dihydrate were purchased from Quimivita (Quimivita, S.A., Spain), and certified as released by the Quality Assurance Laboratory at Deplacén.

Simulated challenge study

Three flasks of each Melagenina® Plus and the Melagenina® lotion lots included in the study were taken. For each product, one flask was provided with RPMI-1640 culture medium supplemented with 10 % fetal bovine serum (FBS; 1:5 v/v), another with medium supplemented with FBS (1:10 v/v) and the third remained as control. All the samples were assayed in triplicates, identified with a code and further delivered to the Chemical Control Laboratory of the Quality Assurance Vice-Direction at Deplacén, for chemical, microbiological and biological control assays as established for industrial scale productions.

The melanocytopoietic activity was determined by the methodology described by Martínez *et al*. [10]. Groups of three C57BL/6 male mice, with a weight of 20 to 22 g, were treated topically in the ears for five consecutive days. They received either lot of the product previously exposed to the treatments described, or the placebo (excipient without API). At 72 h of the last application, animals were sacrificed, ears epidermis samples were taken and processed by the L-Dopa histochemistry technique.

Viral models

Viral models were selected according to its similarity with the possible viral contaminants in the placentas, resistance to physical and chemical agents, and its relevance for being transmitted by blood and its derivatives. They comprised enveloped and non-enveloped viruses, either RNA or DNA genome, respectively: the human immunodeficiency virus type 1 (HIV-1), the bovine viral diarrhea virus (BVDV), the porcine herpes virus type 1 (PHV-1) and the canine parvovirus (CPV) (Table 1).

The high-titer control strains were produced from the controlled cell lines at Lisida, and using culture media manufactured and released by the Cell Culture Laboratory at Lisida (Table 1). Each viral strain was amplified into its cellular specific substrate at a multiplicity of infection of 0.3.

Viral inoculums were titrated by the microtitration method in 96-well plates, according to Johnson and Byington [11]. The median tissue culture infective dose ($TCID_{50}$/mL) was determined as the cytopathic effect, according to the Reed and Muench method [12]. For the HIV-1, the p24 antigen was quantified using the DAVIH Ag p24 kit (Davihlab, Cuba).

Cytotoxicity study

The Melagenina® Plus, Melagenina® lotion, and the purified water solutions of 71 % alcohol and 1 mg/mL $CaCl_2$ were treated in triplicates to decrease its cytotoxicity on each cellular substrate. One volume of each sample was diluted in two and four volumes of a buffered salt solution (BSS), pH 7.2. Serial 1/4 dilutions were made, further adding the cellular substrates at the given concentrations: 5×10^5 cells/mL for the cell line MT4, and 2×10^5 cells/mL for the other cellular substrates). Dilutions were incubated at 37 ºC in a wet atmosphere of 5 % CO_2. Four days later, cultures were observed under an inverted microscope and the lowest toxic dilution of samples were determined, compared to the cell culture controls without samples. The dilution showing moderate to minimum cytotoxicity was set as working dilution for sample processing.

Interference study

The viral models were added to citotoxicity samples at the working dilution, and cells were further homogenized. Two samples of each were taken, one after mixing (M1) and a second (M2) after incubation for 1 h at room temperature. A control sample of each viral inoculum was included, being diluted in a supplemented medium and subjected to the same treatment. The assay was made in triplicates.

Subsequently, samples were processed according to results of the cytotoxicity study, with serial dilutions similar to those used to titrate viral inoculums.

Table 1. Viral models selected for the validation study

Viral model [a]	Genome / Enveloping	Size (nm)	Cellular substrate / Culture media	Resistance to Inactivation	Viral types
HIV-1	RNA / enveloped	80 - 100	MT4 / RPMI-1640	Low	Human retroviruses
BVDV	RNA / enveloped	45 - 60	MDBK / MEM	Moderate	Hepatitis C virus and enveloped RNA viruses
PHV-1	DNA / enveloped	150 - 200	Vero C-1008 / MEM	Low to moderate	Hepatitis B virus and enveloped DNA virus
CPV	DNA / non-enveloped	18 - 26	LFBC / MEM	Very high	Parvovirus B19, hepatitis A virus, non-enveloped DNA and RNA viruses

[a] Viral models: HIV-1: human immunodeficiency virus type 1 (strain IIIB); BVDV: bovine viral diarrhea virus (strain NADL); PHV-1: porcine herpes virus type 1 (Sthendal strain); PVC: canine parvovirus (native strain No. 7164).
MT4: human T-cells line transformed by co-culture with lymphocytes carrying HTLV-1.
MDBK: bovine kidney cell line.
Vero C-1008: green monkey (Sabaeus monkey) kidney cell line (clone 1008).
LFBC: línea celular de fibroblasto de curiel.

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Viral interference was set as the viral titer reduced more than two logs.

Inactivation during storage of Melagenina® Plus

For the challenge at the storage step, a flask from each selected Melagenina® Plus lot was selected. These represents the 0.03 % of the flasks conforming an industrial lot, which were infected with the viral models at a proportion of 1:5 or 1:10 (v/v) according to the simulated challenge study results. Immediately after the addition of each viral model to the flask, a sample was taken, considering it as the initial viral load (VL_i), and flasks were stored at room temperature.

The inactivation kinetics was determined for enveloped viruses monitoring on days 1, 2, 3 and 7 of storage. The VL assessed on day seven was considered as the final VL (VL_f). The study was carried out in triplicates.

The following controls were included:

- Viral strain in 1 mg/mL $CaCl_2$ and in 71 % ethanol, both prepared with purified water.
- Viral strain in supplemented culture media (stored at room temperature, 4 and -85 ºC).
- A flask of Melagenina® lotion, to check if the precipitate observed in the Melagenina® Plus once added the $CaCl_2$ had any incidence in the inactivation property at storage.

Control samples were kept at the same storage temperature of Melagenina® Plus and VLs were quantified at the same time intervals.

Reduction factor calculation

Reduction factor (RF) at storage was calculated according to the following equation [9, 12]:

$$FR = \log \left[\frac{(V_1 \times CVi)}{(V_2 \times CVf)} \right]$$

Where:

V_1 and VL_i: are the initial volume and viral load of the sample; V_2 and VL_f: are the final volume and viral load of the sample.

The process step was classified as effective, moderately effective or ineffective, according to the viral reduction logarithms guidance of the Committee for Proprietary Medicinal Products [9].

Statistical analysis

Viral challenge results were analyzed with the aid of the Statgraphics Plus program, (Statpoint Technologies, Inc., USA; version 5.0, 2000). A Student's t-test was used for paired data, to identify statistically significant differences between VL_i and VL values at different time intervals for each viral model.

Results and discussion

Simulated challenge

Addition of the supplemented media, at a proportion of 1:5 (v/v), to the Melagenina® Plus and the Melagenina® lotion, significantly modified ($p < 0.01$) several quality parameters of these products, such as: ethanol concentration, absorption, cholesterol, nitrogen and proteins concentrations. Therefore, they were not accepted, and the viral challenge was not carried out with that proportion of supplemented media.

When the medium was added at half the proportion 1:10 (v/v), some physicochemical parameters were altered, but not significantly: absorption, evaporation residues, cholesterol, nitrogen and protein concentrations (Table 2). Lipids concentration remained within the established acceptance limits. This parameter was regarded as essential for the melanocytopoyetic activity of placental extracts [2, 13].

Melagenina® Plus diluted 1:10 (v/v) in culture medium showed no significantly different biological activity in mice melanocytes, compared to animals treated either with Melagenina® or the control lot of the product (Table 2). Therefore, these results were accepted, and the 1:10 (v/v) medium proportion was set for viral challenge at storage.

Key aspects are considered in the viral validation study: the proper design of the production process at laboratory scale and results from the simulated challenge, to demonstrate the influence of the culture media used to dilute infectious agents on the physicochemical and biological parameters established for the product [14-16].

Cytotoxicity study

Samples' cytotoxicity were mild to moderate (Table 3), with samples diluted 1:2 (v/v) in BSS showing mild to moderate cytotoxicity, while those diluted 1:5 (v/v) showed minimal changes at best in cell cultures. No differences were observed neither between the two

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Table 2. Results from the quality control of the Melagenina® lotion and the Melagenina® Plus lots in the simulated challenge study at the storage step*

Product	Flask[a]	pH	Alcohol (%)	Density (g/mL)	Minimum absorption (UA)	Maximum absorption (UA)	Evaporation residues (g/100 mL)	α amino acids (mg/100 mL)	Cholesterol (mg/100 mL)	Lipids (mg/100 mL)	Nitrogen (mg/100 mL)	Proteins (mg/100 mL)	Calcium concentration (mg/100 mL)
Melagenina® lotion L-01007	1	7.34	83	0.85	206:1.31	257:0.16	0.33	20	15[b]	85.76	10.68	66.72	np
	2	7.87	74	0.85	204:3.8[b]	nd	0.04[b]	30	15.5[b]	121.2	9.8	61.25	np
Melagenina® Plus L-01034	1	6.28	83	0.85	205:1.33	257:0.2	0.55	30	13[b]	106.1	9.66	60.4	0.36
	2	6.94	74	0.86	204:1.65	255: 0.01	0.725	40	12.5[b]	90.91	24.43[b]	152.7[b]	0.4
Melagenina® Plus 02003	1	6.37	83	0.85	206:1.36	257:0.19	0.465	20	34	90.91	10.33	64.53	0.35
	2	7.07	74	0.856	204:4.9[b]	nd	0.615	30	32.5	90.91	8.22	51.4	0.36

* Values represent means of three replicates per flask. All assayed conditions fulfilled organoleptic properties' standards and showed significant differences in the biological activity assay.
[a] Flask 1: control; Flask 2: media 1:10 (v/v); [b] altered parameter.
nd: not determined; np: does not proceed.

lots of Melagenina® Plus nor of any of them to the one of Melagenina® lotion. Such results were determinant for the selection of the 1:2 (v/v) BSS dilution as the procedure to decrease samples' cytotoxicity.

Several methods have been reported to decrease cytotoxicity (dialysis, gel filtration chromatography, precipitation and serial dilutions); but either the method, special care must be taken to do not deteriorate viral titers, which is detrimental for detecting low VLs [9, 15, 16].

Viral interference study

Viral interference was determined by decreasing or not of the viral models titer in samples, in comparison with the control models (Table 4). The titer decrease for viral controls diluted 1:10 (v/v) in supplemented culture medium did not achieved 1 log.

When adding the enveloped virus models to placental extracts, a fast decrease in viral titers of 2 or more logs was observed, significantly different from the viral control ($p < 0.05$). After 1 hour of incubation, baseline VLs were detected, with highly significant differences ($p < 0.01$). A very similar behavior was observed for the viral control dissolved in 71 % ethanol solution (Table 4). No significant differences were detected between CPV viral titers, regardless ethanol.

These results are in agreement with those from other groups, reporting a marked susceptibility of enveloped viruses to lipidic solvents, and particularly to alcohol concentrations higher than 70 %, but not of the non-enveloped viruses [6, 17, 18].

These results demonstrate that placental extracts do not interfere in the replication of these viral models. The viral interference study provided evidences on the action of the raw materials and the biological products over the viral models' titers, as well as the selection of adequate viral models to study the viral clearance capacity of the Melagenina® Plus production process.

Viral inactivation capacity of Melagenina® Plus during storage

The behavior of enveloped model viruses during the storage of Melagenina® Plus are shown in figure 1. There were no statistically significant differences among viral titers for both lots of Melagenina® Plus and the controls used in the study (Melagenina® lotion and 71 % ethanol solution). Significant differences ($p < 0.05$) were found in VL_i for samples containing 71 % ethanol compared to viral controls, in agreement with results from the viral interference study.

The three viral models followed the typical viral behavior seen for viruses of low resistance to organic solvents, characterized by a sharp drop of titers and complete inactivation within a short period. This last property is related to their relative ethanol resistance. In all the cases, very highly significant differences were detected between VL_i and VL assessed after 24 h ($p < 0.001$) (Figure 1).

PHV-1 was completely inactivated in both infected placenta extracts during the first 24 h of storage, and also in the 71 % alcohol solution (Figure 1). RNA viruses showed a more delayed inactivation, for HIV-1 achieved after 24-48 h of storage with the Melagenina® lotion and 48-72 h after storage with both lots of Melagenina® Plus.

Table 3. Results from the cytotoxicity study for samples further challenged in the cellular substrates*

Samples	Dilution in BSS	Toxicity in the cellular substrates			
		MT4	Vero 1008	MDBK	LFBC
$CaCl_2$ (1 mg/mL)	1:2	Minimal	Minimal	Minimal	Minimal
	1:4	Non toxic	Non toxic	Non toxic	Non toxic
Ethanol 71 %	1:2	Mild	Mild	Mild	Mild
	1:4	Minimal	Minimal	Minimal	Minimal
Melagenina® lotion L-01007	1:2	Mild	Mild	Mild	Mild
	1:4	Minimal	Mild	Minimal	Non toxic
Melagenina® Plus L-01034	1:2	Mild	Moderate	Mild	Mild
	1:4	Minimal	Minimal	Minimal	Minimal
Melagenina® Plus L-02003	1:2	Moderate	Moderate	Mild	Mild
	1:4	Minimal	Minimal	Minimal	Minimal

* Results represent the mean behavior of three replicates per dilution. Toxicity is expressed for each dilution (v/v) of the viral titration system: minimal (1:4), mild (1:16), moderated (1:64) or high (1:256).
SBS: Balanced salt solution.
MT4: human T-cell line transformed by co-culture with lymphocytes carrying HTLV-1.
Vero C-1008: green monkey (Sabaeus monkey) kidney cell line (clone 1008).
MDBK: bovine kidneys cell line.
LFBC: hamster fibroblast cell line.

BVDV showed an intermediate inactivation progression, achieved within the first 24 h of storage with the Melagenina® Plus and after 48 h of storage in Melagenina® lotion and 71 % alcohol. None of the three enveloped virus models showed significant differences in VL compared to controls at any time points.

In the case of BVDV, it is affected by lipid solvents and chemical detergents, but tends to stabilize with the addition of proteins [15, 19]. Nevertheless, the BVDV inactivation observed was not related to the amount of lipid precipitates in the placental extracts, due to the short storage periods assayed.

There were differences in the titers of enveloped virus controls stored at different temperatures. Viral titers decreased in those stored at room temperature, but not at 4 or -85 ºC (Figure 1). For HIV-1, viral titer showed statistically significant differences ($p < 0.05$) only after seven days of storage at room temperature, compared to the titer of the viral control stored at 4 ºC. Such differences were detected for PHV-1 since the third day ($p < 0.05$) and maintained until the seventh. Differences were detected earlier for BVDV, starting at 24 h after storage at room temperature ($p < 0.05$),

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Table 4. Results from the study of viral interference for placental extracts, for viral models used in viral validation†

Viral model[a]	Viral control (1:10)[b]		Ethanol 71 %		Lotion M L-01007[b]		M Plus L-01034[b]		M Plus L-02003[b]	
	M1	M2	M1	M2	M1	M2	M1	M2	M1	M2
VIH-1[c]	6.20	6.13	4.21*	1.3**	4.01*	1.3**	3.24*	1.3**	3.96*	1.3**
VDVB	6.14	5.82	3.31*	1.3**	3.63*	1.3**	3.2*	1.3**	3.81*	1.3**
VHP-1	6.85	6.51	4.11*	1.3**	4.21*	1.3**	3.65*	1.3**	3.91*	1.3**
PVC	7.92	7.92	7.22	6.89	7.25	6.86	7.32	6.57	7.20	6.85

† Values are presented as mean of three replicates for each assay, together with the statistic significance (*$p < 0.05$,**$p < 0.01$).
[a] Viral models: HIV-1: human immunodeficiency virus type 1; BVDV: bovine viral diarrhea virus; PHV-1: porcine herpes virus type 1; CPV: canine parvovirus.
[b] Data expressed in log $TCID_{50}$/mL.
[c] Mean viral titer over the quantified by cytopathic effect and ELISA Ag p24.
Lotion M: Melagenina® lotion.
M Plus: Melagenina® Plus.
M1: sample homogenized just after the addition of each viral model to each cellular substrate, at the dilution determined in the simulated challenge study; M2: sample taken after the incubation of the viral model with the cellular substrate for 1 h at room temperature.

and becoming highly significant at 48 h ($p < 0.01$) and sustained until day seven.

Similar results were reported by Ruibal *et al*. [20], while validating an intravenous immunoglobulin production process. They observed a drop in BVDV titers below 6 logs when stored at 21 ºC for 21 days, while storage at 4 ºC did not affect titers.

The decrease of viral titers for controls stored at room vs. low temperature demonstrated that room temperature storage reinforces the virus inactivation capacity of the product.

The inactivation kinetics of viral models in $CaCl_2$ did not differed from the viral control at room temperature, except for the statistically significant differences ($p < 0.05$) detected for PHV-1 (Figure 1). This indicates that the drop in viral titers was caused by the storage temperature.

The inactivation kinetics for the non-enveloped virus model (CPV) is shown in figure 2. After 24 h of storage, a highly significant drop in viral titers was detected for all the samples in 71 % alcohol, compared to the respective VL_i values. The VL values stabilized borderline to the detection limit of the assay after 48 h, and remained steady until day seven, with very highly significant differences ($p < 0.001$). This viral model was fully inactivated in the Melagenina® lotion on day 14, and after 21 days in both lots of Melagenina® Plus and 71 % alcohol (Figure 2).

Titers of controls stored at 4 and -85 °C remained as initially determined during the whole experiment. They dropped for the room temperature storage since day seven, becoming progressively significant on day 14, highly significant on day 21 and very highly significant on day 28 ($p < 0.001$), this last accounting for a 5.41 logs decrease.

The CPV inactivation kinetics in 1 mg/mL $CaCl_2$ behaved very similar to the viral control stored at room temperature, evidencing the lack of inactivating capacity for $CaCl_2$ at the assayed concentration and reinforcing the conclusion that temperature was the key parameter leading to virus inactivation.

All these results corroborate the effectiveness of the experimental design to study the capacity for viral inactivation during storage of the production process of the placental extract Melagenina®. In fact, enveloped viruses were inactivated within a short period (24 h), with the non-enveloped CPV showing a marginal VL at 72 h of storage [7].

Since CPV, a highly resistant virus, was completely inactivated upon storage, safety was increased both for Melagenina® Plus and its production process. Therefore, CPV results could not be considered relevant just for the non-enveloped viruses, but also this virus can be regarded as a non-specific viral model which provides evidences for the inactivation of new or unpredicted viral contamination in raw materials [7, 9, 15].

Table 5 shows RFs of both placental extracts upon storage, and their respective times for inactivating the challenge VLs. Consequently, enveloped virus were quickly inactivated, after three and four days of storage for Melagenina® Plus and Melagenina® lotion, respectively, without significant differences. The non-enveloped viral model was inactivated after 14 and 21 days of storage for Melagenina® and Melagenina®

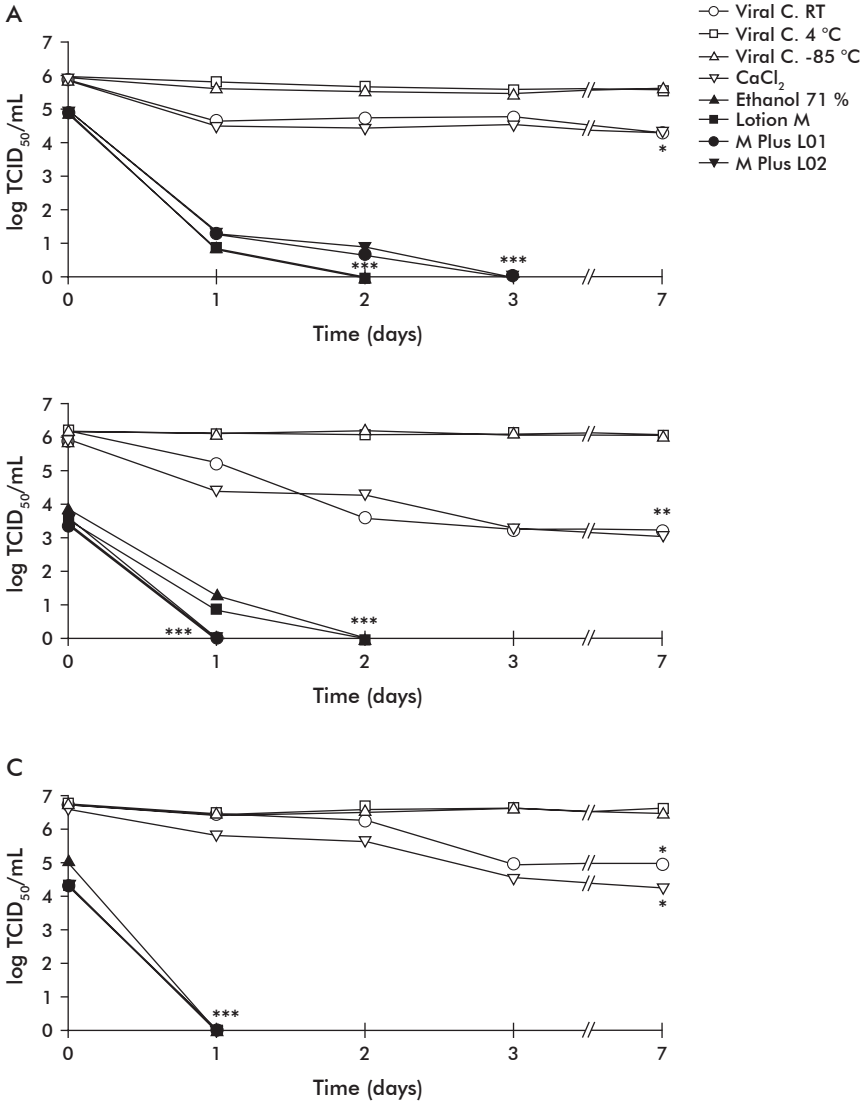

Figure 1. Inactivation kinetics of the enveloped virus models: human immunodeficiency virus type 1 (A); bovine viral diarrhea virus (B) and porcine herpes virus type 1 (C) during the storage of the placental extract (n = 3). Viral C. RT: viral control at room temperature; Viral C. 4 °C: viral control at 4°C; Viral C. -85 °C: viral control at -85 °C; $CaCl_2$: 1 mg/mL calcium chloride; Lotion M: Melagenina® lotion; M Plus L01: Melagenina® Plus Lot 01034; M Plus L02: Melagenina® Plus Lot 02003. Statistically significant differences are indicated (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

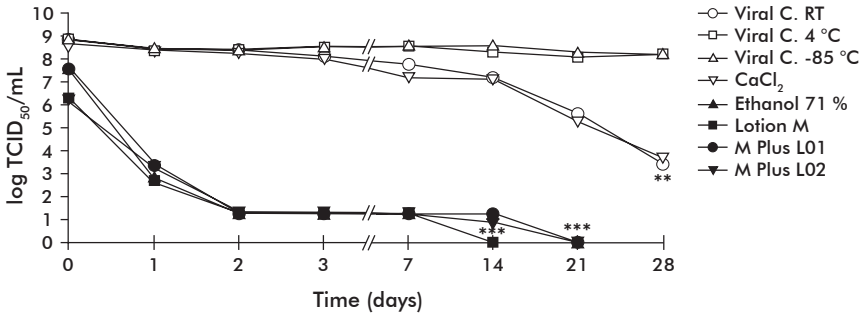

Figure 2. Inactivation kinetics of the non-enveloped CPV model virus in Melagenina® Plus at storage (n = 3). Viral C. RT: viral control at room temperature; Viral C. 4°C: viral control at 4 °C; Viral C. -85 °C: viral control at -85 °C; $CaCl_2$: 1 mg/mL calcium chloride; Lotion M: Melagenina® lotion; M Plus L01: Melagenina® Plus, lot 01034; M Plus L02: Melagenina® Plus, lot 02003. Statistically significant differences are indicated (**$p < 0.01$, ***$p < 0.001$).

Plus, respectively, with very highly significant differences ($p < 0.001$) for each placental extract.

Very highly significant differences ($p < 0.0001$) were observed between the times for inactivation of enveloped and non-enveloped viruses. The difference on time required for viral inactivation in both placental extracts of Melagenina® Plus was related to the presence of greater amounts of lipid precipitates, which could form lattices that protect viruses from the action of the inactivating agent, and, therefore, increase the time required for full inactivation.

Very similar results were observed when validating the placental extract EP-100 with the enveloped virus models, but not for the non-enveloped viruses. These last required a longer storage period (63 days) for complete inactivation [21]. It was related to the storage temperature (4 ºC) and the ethanol concentration in the EP-100 extract. That results corroborate that these two factors are fundamental for the safety of the Melagenina® Plus placental extract.

FRs higher than 4 logs were attained except for the BVDV, and the four viral models were effectively inactivated during the storage step of Melagenina® Plus. The challenge VLs of either enveloped or non-enveloped viruses were fully inactivated, regardless variations in the parameters for this step (Table 5).

The low resistance of the enveloped model viruses to ethanol at the different steps matches that of viral validation studies of different processes for biologicals' production, being closely related to the conditions for the action of the inactivating agent (medium properties, temperature, humidity and pH) [7, 22, 23].

Table 5. Reduction factors of viral models upon storage of placental extracts of Melagenina® lotion and Melagenina® Plus†

Viral models[a]	Lotion M L-01007 RF	Lotion M L-01007 Time (days)	M Plus L-01034 RF	M Plus L-01034 Time (days)	M Plus L-02003 RF	M Plus L-02003 Time (days)
HIV-1[b]	4.96	1	4.96	3	5.06	2
BVDV	3.50	2	3.44	2	3.61	1
PHV-1	4.32	1	4.21	1	4.27	1
CPV	6.35	14***	5.51	21***	6.09	21***

† Values are presented as the mean of three replicates per assay. Inactivation time of the non-enveloped virus was very highly significant (*** $p < 0.001$).
[a] Viral models: HIV-1: human immunodeficiency virus type 1; BVDV: bovine viral diarrhea virus; PHV-1: porcine herpes virus type 1; CPV: canine parvovirus.
[b] Mean viral titer for values calculated by the cytopathic effect method and the p24 Ag ELISA.
Lotion M: Melagenina® lotion.
M Plus: Melagenina® Plus.
RF: Reduction factor.

The virucidal action of ethanol is affected by low temperature and high protein and lipid concentrations, these factors stabilizing viruses and making them more resistant to the adverse conditions of the culture medium and disinfectants [7, 18, 22, 23]. Both culture medium and ethanol were absent during placental extract storage, favoring the inactivation of all the viral models while validating the production process.

In summary, the Melagenina® Plus formulation in alcohol solution of 71 % or higher, together with its storage at room temperature, supported the complete inactivation of all the viral models tested, regardless its resistance to inactivating agents. It provided an adequate safety level to the production process, being able of eliminating new or unpredictable viral contaminations of placentas.

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