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An Alternative *In Vitro* Methodology for the Quality Control of Equine-Chorionic Gonadotropin in Commercial Products

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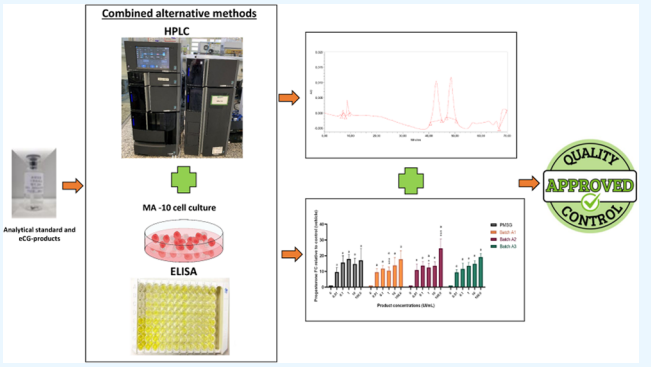
ABSTRACT: Equine-chorionic gonadotropin (eCG) is widely used in fixed-time artificial ovulation and superovulation protocols to improve reproductive performance. Commercial products available in the market for veterinary use consist of partially purified preparations of pregnant mare serum gonadotropin (PMSG). eCG is a heterodimeric glycoprotein and is thus extremely challenging to quantify through chromatography. Pharmaceutical companies usually carry out *in vivo* methods to measure the potency of finished products containing eCG. Considering the three Rs principle (refinement, replacement, and reduction), the aim of this study was to develop *in vitro* assays using high-performance liquid chromatography (HPLC) and cell culture techniques to predict the biological activity of products containing eCG. The experimental conditions established for the chromatographic method allowed an efficient separation between the eCG peaks and the excipients present in the formulation, with a consistent chromatographic profile between batches of eCG-products and the analytical standard (PMSG). In cell culture, stimulation of MA-10 cells with both PMSG and products containing eCG resulted in significant progesterone production in all tested concentrations and a similar profile between the products and control, indicating substantial biological activity. The data presented corroborate the potential use of the combination of the chromatographic profile and cell culture method for the successful quality control of commercial preparations containing eCG. Beyond the practical benefits of reduced time and cost, these approaches align with a growing recognition of the need to reduce the reliance on animal models for the quality control of products containing eCG.



1. INTRODUCTION

The equine-chorionic gonadotropin (eCG), previously known as pregnant mare serum gonadotropin (PMSG), is a glycoprotein secreted in high concentration by trophoblastic cells present between the 36th and 120th days of gestation in mare.[1,2] Protein analyses show that eCG is a heterodimer composed of α and β subunits and, similarly to pituitary gonadotropins, is post-translationally glycosylated.[2] Both chains present biological activity, however, while the α portion is common to all glycoprotein hormones, the β chain binds to its specific receptor.[3] eCG has luteinizing hormone (LH)-like activity in horses, but variable LH- and follicle stimulating hormone (FSH)-like function in other species, stimulating the accessory corpus luteum to produce progesterone to support early gestation.[1−3] Considering this dual role, eCG is a valuable treatment broadly used in veterinary medicine to induce artificial reproductive cycles and out-of-season breeding, especially in ruminants, inducing ovulation and synchronizing estrus.[4,5]

To ensure the biological activity and quality of commercial eCG products, pharmaceutical companies use *in vivo* methods, such as measuring ovarian weight gain in treated rats.[6] Regarding ethical and economic aspects, this technique consumes a large number of animals and resources, and the results of eCG treatment are often inconsistent, resulting in variable stimulation and ovarian weight gain among animals.[7,8] The shift in the quality control concept from animal experimentation to alternative methods follows the three Rs principles (replacement, reduction, and refinement) recognized by various competent international organizations.[9] Thus, replacing the use of animals for *in vitro* assays is considered an attractive alternative for determining eCG potency.

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Despite the evident need, to our knowledge, there are few reports showing partially or completely replacement of the use of animals for the batch control of finished products containing isolated eCG. The use of physicochemical and cell culture approaches has been reported in the literature as potential alternatives to determine the biological activity of different proteins, including glycoprotein hormones, with greater precision and accuracy than conventional methods.[10,11] MA-10 cell, a mouse cell line derived from the testis of a transplantable Leydig tumor cell, has previously been shown to synthesize increased amounts of progesterone under LH or human CG (hCG) stimulation, which could represent an *in vitro* bioassay to replace eCG potency *in vivo* tests.[12] The development of a chromatographic analysis for the qualification and quantification of the eCG is challenging, especially to discriminate the form of the intact heterodimer, the α and β subunits, forms without biological activity, impurities, and to detect structural changes related to loss of potency in the products.[10,13] In this paper, we aimed to establish a combination of *in vitro* assays that could enable the monitoring of batch-to-batch quality and consistency.

2. MATERIALS AND METHODS

2.1. Preparation of Products and Reagents. Five (for cell culture assays) or four (for chromatographic analysis) batches of commercial products containing eCG from two different suppliers and the analytical standard PMSG (2035.4 IU/vial, Ningbo Second Hormone Factory, batch 2101) were used to develop the methodologies. The biological activity of all batches was previously approved by the *in vivo* quality control method, which measured ovarian weight gain in the treated rats.

For chromatographic analysis, commercial products and the analytical standard PMSG were diluted according to the manufacturer's recommendations in sterile water to a final concentration of 2000 IU/mL. The mobile phase was composed of a mixture of ammonium phosphate buffer (0.05 M, pH 8.6) and acetonitrile (99.9% purity, Merck, batch I1212230). An ammonium phosphate buffer was prepared from a combination of monobasic ammonium phosphate (98% purity, J.T.Baker, batch A07C58) and dibasic ammonium phosphate (98% purity, Synth, lot 236854) and adjusted to the final pH value with ammonium hydroxide (30% purity, J.T.Baker, batch 0000229973).

Similarly, for cell culture assays, PMSG and commercial products were diluted in sterile water according to the manufacturer's recommendations to obtain a stock solution of 2000 IU/mL. For cell treatment, the stock solution was serially diluted in DMEM/F-12 without supplementation to the final concentrations of 0.01, 0.1, 1, 10, and 100 IU/ml eCG.

2.2. Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC). Analytical RP-HPLC runs were carried out using a C4 column (Jupiter Phenomenex, 250 mm × 4.6 mm ID., 300 pore size, 5 μm particle size) connected to an HPLC system (Shimadzu model LC-40D) equipped with an ultraviolet (UV) detector (SPD-M40). The solutions used to develop the gradient were (A) ammonium phosphate buffer (0.05 M, pH 8.6) and (B) acetonitrile. The solvent gradient for eCG elution, in volumetric ratios of solvents A and B, was first 5 min in 90A/10B, followed by 45 min in 60A/40B and 90A/10B in the last 20 min. Total run time was 70 min. Aliquots of 50 μL for commercial products and PMSG were processed at a flow rate of 0.4 mL/min. Column oven temperature was kept at 25 °C, and the detection was by UV absorbance at 220 nm. The Empower software was used to analyze the quality of separation and quantify the different parameters.

2.3. Collection and Analysis by Advanced Mass Spectrometry. Initially, the batch B1 sample was diluted in sterile water according to the manufacturer's recommendations to a final concentration of 6000 IU/mL. Subsequently, three chromatographic runs were performed following the chromatographic conditions previously described. The peak corresponding to the retention time of approximately 57 min observed in the chromatogram of this product was collected and approximately 100 μL corresponding to 50 μg was been quantified, using the Bradford method with the Protein Assay Dye Reagent Concentrate (Bio-Rad, code 500−0006).[13] The sample was initially dried in a SpeedVac Concentrator (50 μg) (Savant SPD121P, Thermo Scientific) and resuspended in 0.05 M Tris−HCl buffer, pH 8.8, containing 8 M urea. Then, the sample was subjected to the reduction of protein disulfide bridges by the addition of 50 μg of dithiothreitol (DTT) and incubation for 1 h at 37 °C, followed by alkylation with 250 μg of iodoacetamide and incubation for 1 h at room temperature, in the dark. The resulting sample volume was diluted 5-fold with 0.05 M Tris−HCl solution, pH 8.8, and incubated with 1 μg of trypsin (Promega, V511A) at 37 °C overnight. Prior to applying the samples to the mass spectrometer, the samples were cleaned/desalted using the OASIS HLB Cartridge 1 cm^3 column (cat. number: 186000383, Waters), as described by the manufacturer.

The sample prepared as described above was analyzed using a state-of-the-art mass spectrometer, the Orbitrap Eclipse (ThermoFisher), coupled to a Nanoflow LC-MS/MS chromatography system (Dionex Ultimate 3000 RLSCnano System, ThermoFisher). Approximately, 1 μg of the sample was injected into the sample for analysis. The peptides were separated on a Nanoease MZ peptide BEH C18 column (130, 1.7 μm, 75 μm × 250 mm, Waters) at a flow rate of 300 nL/min using a 4−50% acetonitrile gradient in 90 min. Data acquisition on MS1 covered the M/Z range of 375−1500 (120,000 resolution, AGC target of 1E6, maximum injection time of 100 ms). The most abundant ions were selected for MS/MS with a collision energy of 30%, a precursor isolation window of 1.2 m/z, an AGC target 1E5, and a resolution of 15,000. Raw data files (.raw) were converted to mzXML format and processed using the Comet algorithm (v. 2018). The Uniprot database for horses was used. Peptides meeting a 3% maximum error criterion, as determined by Peptide Prophet, were used for intensity extraction with the Xpress algorithm. The peptides and their intensities were grouped to generate the protein intensity using a script in the R language.

2.4. Mass Spectrometry. The molecular mass of the samples was determined using a MALDI-TOF/TOF UltraFlex II mass spectrometer (Bruker Daltonics), operated in positive linear mode. A saturated solution of the DHAP matrix (2,5-dihydroxyacetophenone) in ethanol was prepared. Then, 1 μL of this matrix was mixed with 1 μL of the sample solubilized in a purified H_2O:Acetonitrile (7:3) solution containing 0.1% TFA (trifluoroacetic acid). After mixing, 1 μL was spotted on the MALDI plate, and after drying, the plate was introduced into the equipment for analysis. The equipment was previously calibrated with Peptide Standard II from Bruker Daltonics. The obtained mass spectra were analyzed by using Flex Analysis v3.3 software.

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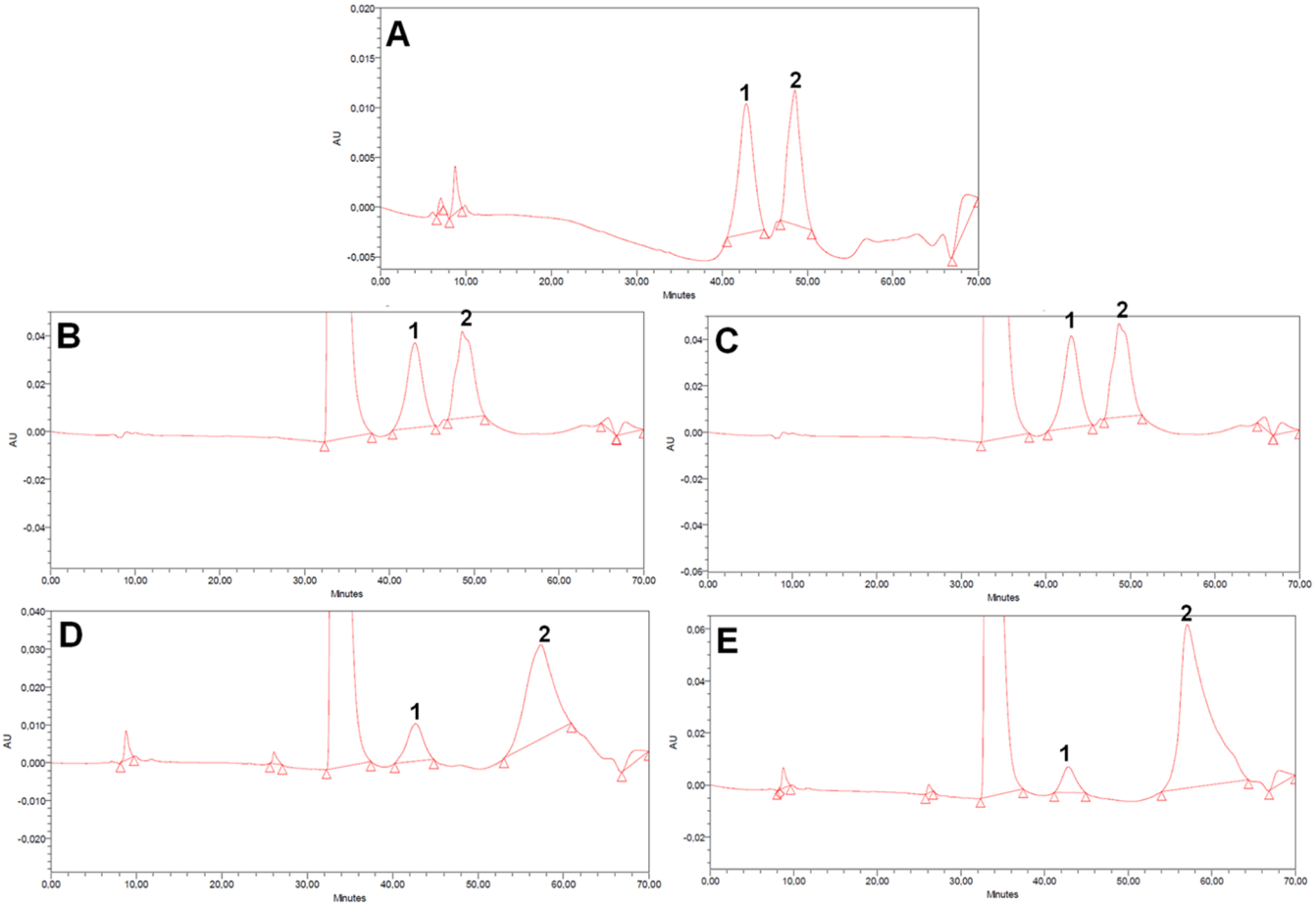

Figure 1. Chromatographic profiles of the analytical standard and different batches of commercial products containing eCG hormone from two different suppliers A and B on reversed-phase HPLC: (A) PMSG; (B) Batch A1; (C) Batch A2; (D) Batch B1; and (E) Batch B2. The number above the chromatograms highlights the peaks of interest analyzed. Adsorbed proteins were eluted with a stepwise gradient using solvents (A) ammonium phosphate buffer (0.05 M, pH 8.6) and (B) acetonitrile at a flow rate of 0.4 mL/min (Section 2.2). Absorbance was monitored at 220 nm.

2.5. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the method described by Laemmli (1970).[14] A resolution gel containing 13.5% (m/V) bis(acrylamide/acrylamide), 1 M Tris−HCl buffer, and 0.1% sodium dodecyl sulfate (SDS) was prepared. The concentration gel was prepared with 5% acrylamide in a 0.5 M Tris−HCl buffer and 0.1% SDS. SDS-PAGE was performed under reducing conditions with β-mercaptoethanol. A volume of 12 μL from each 6000 IU/mL sample was applied per well, and sample migration was compared with the molecular mass standard (Precision Plus Protein Standards Dual Color, Bio-Rad #161−0374). Electrophoretic run conditions were 110 V, 40 mA, 15 W, for 2 h and the obtained gels were stained with 0.006% *Coomassie Brilliant Blue* G-250.[15]

2.6. Cell Culture and Treatment. MA-10 cells were purchased from the American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's Modified Eagle Medium F-12 (DMEM/F-12), supplemented with 15% heat-inactivated horse serum, and then incubated at 37 °C in 5% CO_2. For cell viability assays and progesterone quantification, cells were culture in DMEM/F-12 without supplementation and treated with vehicle (sterile water−control cells) or increasing amounts of eCG (0.01, 0.1, 1, 10, and 100 IU/mL) for 24 h. After treatment, supernatants were collected for subsequent progesterone quantification.

2.7. Cell Viability. Cell viability of MA-10 cells was determined by using the (3-(4,5)dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay after treatment with increasing concentrations of eCG. Cells were seeded in flat-bottom 96-well plates at a density of 15,000 cells per well and cultured in complete growth medium overnight. The next day, the complete medium was discarded and cells were treated with eCG or vehicle for 24 h as described above. At the end of the incubation period, supernatants were collected, and the medium was replaced with fresh complete medium containing 0.5 mg/mL MTT, followed by an incubation of 4 h at 37 °C. Living cells reduced MTT to formazan, which was quantified by measuring absorbance at 570 nm. The viability of treated cells was expressed relative to control cells (cells treated with vehicle) and evaluated in 6 well replicates for each concentration. To minimize bias in the determination of progesterone production, wells with cell viability outside the range of 75−125% relative to the mean cell viability of control cells were excluded from further analysis.

2.8. Progesterone Quantification. Progesterone quantification was determined by ELISA in MA-10 cell supernatants after 24 h of stimulation with vehicle or eCG. Supernatants

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were removed from each experimental well (200 μL) and stored at −20 °C until analysis. Cayman's progesterone ELISA kit (item No. 582601, Cayman Chemical) was used according to the manufacturer's instructions to determine progesterone concentrations. Data were expressed as fold changes relative to the control (vehicle).

2.9. Statistical Analysis. Cell viability and progesterone quantification analysis were performed using two-way ANOVA tests to compare the effects of commercial products containing eCG with the analytical standard PMSG and/or control cells (vehicle). A p-value <0.05 was considered significant.

3. RESULTS AND DISCUSSION

Gonadotropins are a class of glycoprotein hormones that play a central role in artificial reproduction and are commonly used in the biopharmaceutical field for the treatment of infertility.[16] In our study, we conducted a comprehensive analysis of the chromatographic profiles of an analytical standard (PMSG) and commercial batches of products containing eCG from two different suppliers (sources A and B). The experimental conditions established for the chromatographic method provided an efficient separation between the eCG peaks and the excipients present in the formulations (Figure 1A−E). The system suitability parameters adhered to the validation standards set forth by the Food and Drug Administration (FDA)[17] (Table 1). Notably, PMSG and most of the peaks observed in the analyzed products exhibited USP Plate Count >2000 and USP Resolution >2 (Table 2). However, peak 2 of PMSG and batches A1 and A2 displayed USP Resolution <2, which can be attributed to the greater asymmetry of these peaks, indicated by USP Tailing >1 (Table 1).

Table 1. System Suitability Determined for RP-HPLC Analysis of the Analytical Standard and Different Batches of Commercial Products Containing eCG Hormone[a]

products	RT (min)	area	USP plate count	USP resolution	USP tailing
PMSG Peak 1	42.813	1.565.108	2796.79	15.50	0.98
PMSG Peak 2	48.508	1.412.805	3274.50	1.73	1.09
Batch A1 Peak 1	42.986	4.615.172	2502.35	3.61	0.96
Batch A1 Peak 2	48.606	4.895.711	2161.20	1.48	1.26
Batch A2 Peak 1	43.001	5.170.577	2488.03	3.56	0.94
Batch A2 Peak 2	48.636	5.502.857	2395.58	1.56	1.28
Batch B1 Peak 1	42.685	1.289.670	2381.88	3.48	0.94
Batch B1 Peak 2	57.306	5.432.001	2391.32	2.99	0.93
Batch B2 Peak 1	42.826	1.087.813	3101.76	3.76	1.13
Batch B2 Peak 2	57.060	14.650.075	2311.02	2.86	1.90

[a]Retention time (RT); peak area, USP plate count; USP tailing, and USP resolution between eCG isoforms or eCG isoforms and excipients.

The analytical standard exhibited two distinct peaks with retention times of 42.813 and 48.508 min, a consistent chromatographic profile with batch A1 and batch A2 (Figure 1A−C and Table 1). In contrast, the characterization of batches B1 and B2 revealed chromatographic discrepancies in comparison to PMSG, highlighting the emergence of a peak in both batches at retention times of 57.306 and 57.060 min (Figure 1A,D,E and Table 1). To elucidate the protein composition of the differentiated peaks observed in batches B1 and B2, in comparison to the analytical standard, and with a retention time of approximately 57 min, a selective collection by chromatography of the peak present in the batch B1 was performed, followed by advanced mass spectrometry. The results allowed us to estimate the percentage of proteins present in the sample, showing that approximately 95% of the total sample corresponded to albumin, as inferred by the peak area analysis (Tables 2 and S1).

Table 2. Proteins Identified in the Peak with a Retention Time of Approximately 57 min Collected from the Batch B1 Product by Advanced Mass Spectrometry Analysis

protein description	percent coverage	total indep spectra	area %
albumin OS = Equus caballus OX = 9796 GH = ALB PE = 4 SV = 1	52	2926	94.64
α-2-HS-glycoprotein OS = Equus caballus OX = 9796 GN = AHSG PE = 4 SV = 1	72	439	2.62
extracellular matrix protein 1 OS = Equus caballus OX = 9796 GN = ECM1 PE = 4 SV = 3	69	198	0.57
β-2-microglobulin OS = Equus caballus OX = 9796 GN = B2M PE = 1 SV = 1	69	98	0.45
Ig-like domain-containing protein OS = Equus caballus OX = 9796 PE = 4 SV = 2	69	5	0.35
Ig-like domain-containing protein OS = Equus caballus OX = 9796 PE = 4 SV = 3	31	51	0.17
Ig-like domain-containing protein OS = Equus caballus OX = 9796 PE = 4 SV = 1	63	45	0.16
lipocalin/cytosolic fatty-acid binding domain-containing protein OS = Equus caballus OX = 9796 GN = LOC100050034 PE = 3 SV = 1	61	48	0.15
vitamin D-binding protein OS = Equus caballus OX = 9796 GN = GC PE = 4 SV = 2	63	128	0.13

In the quality control of pharmaceutical industries, an important issue is the development of methodologies that enable the successful identification of active ingredients without the interference of other substances, such as excipients or contaminants present in pharmaceutical formulations.[18] In this context, we successfully established a chromatographic method for the qualitative identification of eCG contained in commercial products, even in the presence of an excess of albumin. This molecule constitutes approximately 60% of the total proteins in serum and plays a vital role in the transport of other proteins, including eCG, through the bloodstream.[19] Furthermore, albumin is a primary contaminant found in the serum of pregnant mares, the raw material used for the purification and production of eCG-commercial preparations.[20] However, the chromatographic methodology effectively separated eCG and detected variations in the chromatographic profiles of commercial batches without requiring the addition of salts, pH adjustments, or the removal of other proteins that could compromise the biological activity of the compounds—classic strategies widely reported in the literature.[21,22] Notably, the differences observed in chromatographic profiles between the analyzed batches and the analytical standard align with previous studies[20] and can be attributed to several factors, including discrepancies in production and purification processes of the batches, variation in impurities between batches, the specific mare used to obtain the serum, the collection of serum at different gestation

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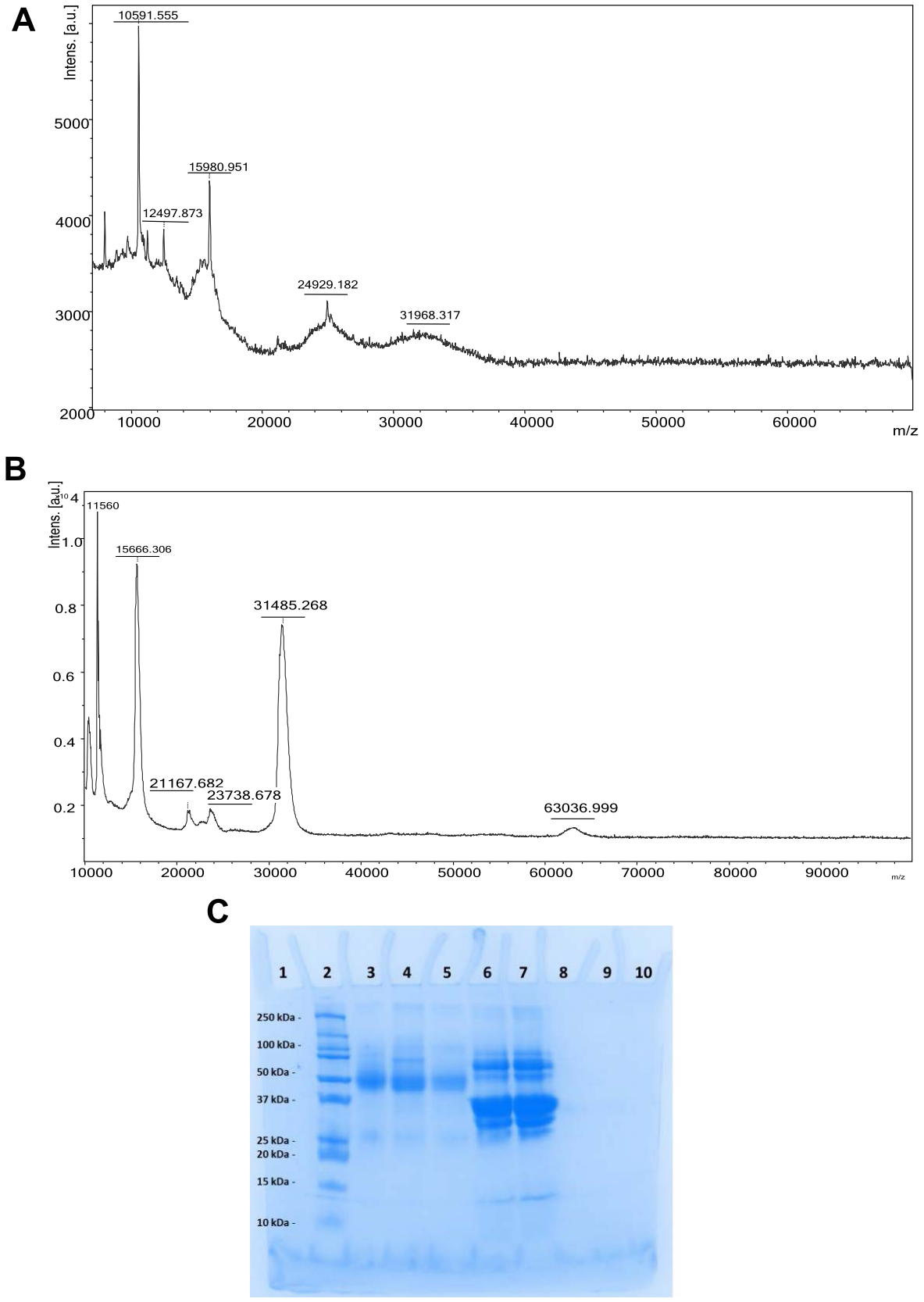

Figure 2. (A, B) Mass spectra of intact products containing eCG obtained by MALDI-TOF (positive linear mode) using 2,5-Dihydroxyacetophenone (DHAP) matrix. (A) Batch A2. (B) Batch B2. (C) SDS-PAGE (13.5%) under reducing conditions was stained with 0.006% *Coomassie Brillant Blue* G-250. Lanes 1−7: Lane 1: Empty; Lane 2: Molecular mass standard (Bio-Rad #161−0374); Lane 3: PMSG; Lane 4: Batch A1; Lane 5: Batch A2; Lane 6: Batch B1; Lane 7: Batch B2. A volume of 12 μL from each 6000 IU/mL sample was applied per well. Electrophoretic run conditions: 110 V, 40 mA, 15 W, for 2 h.

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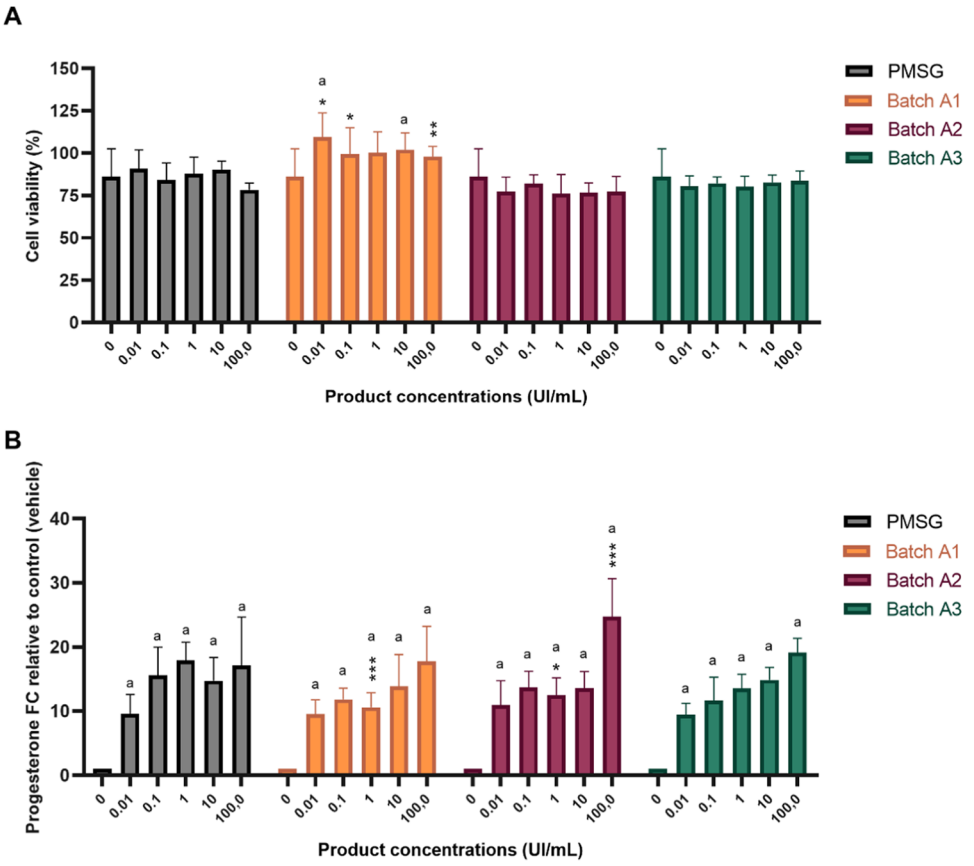

Figure 3. Effects of commercial products containing eCG on the MA-10 Leydig cells. (A) Cell viability was determined by MTT assay after 24 h treatment with increasing concentrations of commercial products containing eCG, the analytical standard PMSG or vehicle (control cells), $N = 6$. (B) Progesterone quantification was determined by ELISA in MA-10 supernatants after 24 h of stimulation with vehicle, PMSG or commercial products containing eCG. Data expressed as fold change (FC) relative to control (vehicle), $N = 6$. For both assays, [a]cell viability compared to control (vehicle). *Cell viability was compared to the same concentration of PMSG. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

periods, as well as differences in the glycosylation profiles of eCG isoforms, which affect hydrophobicity and molecular behavior in the HPLC system.[2,23−25]

In Figure 2, we compared the mass spectra of intact eCG products from different sources (Figure 2A—batch A2 and Figure 2B—batch B2) which reveal similarity in some peaks, but some relevant differences between others. Both sources exhibited a peak with a molecular mass near 31 kDa, along with others close to 24, 10−11 and 15 kDa. Human chorionic gonadotropin (hCG) is known to present peaks with m/z at 13.9, 23.6, and 37.6 kDa, corresponding to the singly charged hCGα-subunit, hCGβ, and intact heterodimer hCG analyzed *via* MALDI-TOF.[26] Thus, the molecular masses observed in the mass spectra of intact eCG-products likely correspond to the intact heterodimer and its subunits. Moreover, it is established that hCG, as well as similar hormones from other species, is highly glycosylated in their structures, accounting for up to 30% of their molecular mass.[27] In the case of eCG, this percentage can reach 45% of its structure.[28] Thus, their subunits are heterogeneous even in their carbohydrate composition. This variability may explain the discrepancies between the molecular masses of the complete products (Figure 2) and between the masses found for the human and equine forms. The SDS-PAGE gel further illustrated these batch differences. A batch comparison revealed a similarity between PMSG and batches A1 and A2, as well as between batches B1 and B2 (Figure 2C). However, upon comparison of these batches from different sources, notable differences were observed in the electrophoretic bands, particularly a more intense band above 50 kDa in batches from source B (Figure 2C). Additionally, a molecular mass corresponding to 63 kDa was identified in the mass spectrum (Figure 2B) and in the SDS-PAGE gel (Figure 2C) for the batches of source B. This molecular mass was not present in the intact product from source A, in agreement with results observed in Figure 1 and in the advanced mass spectrometry analysis (Table 2), suggesting potential contamination in these batches.[29]

Although chromatographic analysis successfully characterized and qualitatively identified eCG in different commercial products, confirmation of its effective biological activity requires additional assessments. Previous studies have demonstrated that the MA-10 Leydig cell line responds to hCG by stimulating progesterone biosynthesis and that eCG exhibits both LH and FSH-like activities in nonequid species.[30] Based on this, we tested whether this mouse-derived cell line could serve as an alternative method to evaluate the biological activity of products containing eCG. For consistency, we limited our evaluation batches from source A due to their similarity with the analytical standard PMSG, used here as a positive control in our bioassays.

To ensure that observed effects on progesterone production were not confounded by changes in cell viability, we first assessed the impact of products containing eCG on MA-10 cell viability. In general, treatment with commercial products and

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the analytical standard PMSG had minimal effects on the cell viability (Figure 3A). Notably, only batch A1 treatment demonstrated increased cell viability at concentrations of 0.01 and 10 IU/ml when compared to the vehicle control and at concentrations of 0.01; 0.1, and 100 IU/mL relative to PMSG (Figure 3A).

Confirming our hypothesis, stimulation of MA-10 cells with both PMSG and products containing eCG resulted in significant progesterone production at all concentrations tested, compared to the vehicle control (Figure 3B). Progesterone production was at least 10-fold higher than that of the control, indicating substantial biological activity. Furthermore, progesterone production patterns in the commercial products closely mirrored those observed with PMSG (Figure 3B), suggesting comparable biological activity. Consistent with these findings, all three commercial batches (A1, A2 and A3) used in our *in vitro* assays were previously approved in the *in vivo* method for their biological activity, as observed by increased ovarian weight in treated rats. To the best of our knowledge, these are the first data showing progesterone production by MA-10 cells treated with eCG for evaluation of quality control of commercial products.

An approach analogous to our bioassay has been employed to screen and evaluate potential endocrine-disrupting chemicals using a human adrenocortical carcinoma cell line (NCI-H295R).[31,32] These cells enable the examination of chemical effects on steroidogenesis, a process quantifiable by the production of 17β-estradiol and testosterone, a method now recognized as an OECD Guideline for the Testing of Chemicals.[32,33] Similarly to our *in vitro* bioassay, the H295R assay is carried out in a multistep process that involves determining cell viability prior to quantifying specific hormones. In both cases, the assessment of the potential impact of the tested products on cell viability allows to discriminate between the effects due to cytotoxicity and those due to the direct effect on hormone biosynthesis.

The biological activity of recombinant eCG isoforms has been investigated in various cell models, such as CHO-K1,[34,35] and parental PathHunter cells genetically modified to express the LH/CG receptor (LHR/CGR),[36] Y1 mouse adrenal cortex tumor cells stably expressing the human FSH receptor, primary rat Leydig cells,[37] primary rat granulosa cells,[38] primary bovine luteal cells,[39] and the mLTC mouse Leydig tumor cell line.[40] However, the final outcome evaluated varied in each study, including assessments of hormone biosynthesis, relative gene and protein expression, and cyclic adenosine monophosphate (cAMP) production, among others. Compared to all these bioassays, the use of the MA-10 cells, a cell line without any genetic modification and commercially available, presents significant advantages. In addition to the practical benefits of reduced time and cost, this approach aligns with the growing recognition of the need to reduce the reliance on animal models for the quality control of products containing eCG.

4. CONCLUSIONS

In this study, we developed a combination of two *in vitro* assays that allow the prediction of quality control and the biological activity of commercial products containing eCG. HPLC analysis proved to be robust to distinguish the chromatographic profiles of commercial batches from different suppliers and analytical standard. Additionally, the progesterone stimulation assays of MA-10 cells with eCG-products provided an effective measurement of biological activity. Together, these methods could help in predicting the approval or rejection of these batches in the routine of quality control processes. Moreover, both HPLC and progesterone stimulation assays in combination, emerge as promising alternatives to predict the biological activity of eCG, potentially replacing traditional *in vivo* bioassay. However, further validation is required for applications of these methods in a quality control routine, which has been progressively introduced by Regulatory Agencies and Pharmacopoeias to replace expensive and imprecise bioassays based on the use of animals.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c10780.

Sequence of identified peptides batch B1—by MS/MS spectrometric analysis (Table S1) (PDF)

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Notes

The authors declare no competing financial interest.

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