TRANSDERMAL FORMULATIONS CONTAINING HUMAN SEXUAL STEROIDS: DEVELOPMENT AND VALIDATION OF METHODS AND *IN VITRO* DRUG RELEASE

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Recebido em 25/06/2013; aceito em 12/12/2013; publicado na web em 27/03/2014

In vitro release of bioidentical hormones in four different liposomal transdermal emulsions (containing testosterone, progesterone, estradiol, or estradiol and estriol) was assessed. For this purpose, novel high-performance liquid chromatography methods were developed and validated in an eco-friendly manner and used to determine the *in vitro* release of such products. The methods were suitable for our intended goal, and the emulsions employed were found to be effective as transporting candidates for the efficient release of hormones in the transdermal delivery of human sexual steroids.

Keywords: *in vitro* drug release; transdermal steroids; semi-solid formulations.

INTRODUCTION

Today, the expression “bioidentical sexual hormones” often refers to steroids that, despite being obtained through organic synthesis using diosgenin (isolated from *Dioscorea villosa* L (also known as wild yam) as a primer substrate, possess a chemical structure identical to human endogenous sexual hormones.^{1,2} Currently, they are considered as an alternative to the all-synthetic and traditional hormone replacement therapy, which has been reported to be harmful and threatening to patients.³ However, despite them being relatively new, the bioidentical hormones in fact comprise well-known human sexual steroids: for instance, testosterone (T), progesterone (P), 17-β-estradiol (E2), and estriol (E3) (Figure 1).

dosage.⁴ Furthermore, their physicochemical properties make them exceptional candidates in the therapeutic field as they can easily diffuse through the human skin, notably because of their low molecular weight and adequate lipophilicity.

Traditional transdermal dosage forms consist of transdermal patch and injectable formulations. However, emulsions have attracted increasing attention over the recent years as they not only mimic clinical performance of a patch but also lower the incidence of skin irritation, thus enhancing the patients' compliance.⁴ As their application assumes the physiological hormonal environment, emulsions are able to exploit this advantage and reduce the side effects and risks associated with hormone therapy.⁵ Despite the numerous advances, transdermal semi-solid pharmaceuticals pose a few specific challenges, e.g., to reach systemic circulation in sufficient quantity to exert their biological activities, the pharmaceuticals have to be applied over a large surface area. To this end, liposomal vehicles have been designed to overcome this drawback,⁶ which aid in delivering human steroids transdermally. The technology also offers the flexibility to alter biopharmacological properties of hydrophobic drugs, thereby increasing their solubility and improving release kinetics and bioavailability.⁷

One of the main methods for assessing the analysis and quality verification of these transdermal gels and creams⁸ is the *in vitro* drug release test, which requires a pre-validated quantification method. This is of crucial relevance for not only industrial emulsions but also compounded counterparts, as compounding pharmacies are significantly responsible for the dissemination of transdermal hormones.

Given this context, this study was conducted to (i) develop, optimize, and validate innovative high-performance liquid chromatography (HPLC) methods for the quantification of bioidentical hormones in four different transdermal emulsions (containing T, P, E2, or E2 + E3) compounded with a liposomal oil-in-water vanishing cream base (Pentravan[®]) as a vehicle; (ii) determine the best physiological receptor medium for these substances for application in *in vitro* drug release tests; and (iii) determine the *in vitro* drug release of such products. To the best of our knowledge, no data has been reported on this. Commonly, reverse-phase HPLC organic solvents such as acetonitrile (ACN) and methanol (MeOH) are generally used to achieve the separation of analytes; however, they are considered

Figure 1. Molecular formulae and chemical structures for the human sexual hormones used in this study.

Remarkably, these molecules have low oral bioavailability, allowing for their efficient systematic delivery through transdermal

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as significant pollutants.⁹ It is important to note that the entire study described herein was developed in an eco-friendly, green-chemistry manner—a current trend in analytical chemistry. To the best of the author's knowledge, this approach has not yet been exploited for the analysis of human sexual steroids.

EXPERIMENTAL

Reagents, reference standard, and materials

Ethanol (EtOH) used in the preparation of the mobile phase was HPLC grade, whereas the other chemicals, including sodium chloride (NaCl), potassium chloride (KCl), calcium chloride (CaCl₂), magnesium sulfate (MgSO₄), magnesium chloride (MgCl₂), sodium sulfate (Na₂SO₄), sodium bicarbonate (NaHCO₃), potassium dihydrogen phosphate (KH₂PO₄), disodium hydrogen phosphate (Na₂HPO₄), acetone, ACN, chloroform, EtOH, ethyl ether, MeOH, and tetrahydrofuran, were of analytical grade and purchased from Sigma-Aldrich (USA). Ultrapure water (H₂O) obtained in an AquaMax-Ultra 370 Series (Young Lin, Korea) (18.2 MΩ cm resistivity at 25 °C and <10 ppb total organic carbon) was used throughout the analysis in the study. T, P, E2, E3, ethoxydiglycol, and Pentravan® (liposomal vehicle) for emulsion compounding were all cordially gifted from Fagron (Brazil). The reference standards used were obtained from United States Pharmacopeia (USA). All mobile phases and receptor media were filtered using a 0.45-μm filter membrane (Sartorius, Germany) and degassed using an ultrasonic apparatus (Cristófoli, Brazil) for 30 min prior to use. All volumetric glassware was calibrated before use.

Transdermal emulsions

The studied transdermal emulsions were compounded and labeled as T_{emuls}, P_{emuls}, E_{emuls}, and Biest ("emuls" is the abbreviation for emulsion) according to the respective active ingredient: T_{emuls}: T–50.0 mg, ethoxydiglycol–0.5 mL, and vehicle in quantity sufficient for (qs) 1 g; P_{emuls}: P–50.0 mg, ethoxydiglycol–0.5 mL, and vehicle–qs 1 g; E_{emuls}: E2–0.5 mg, ethoxydiglycol–0.01 mL, and vehicle–qs 1 g; Biest: E2–1.0 mg, E3–4.0 mg; ethoxydiglycol–0.03 mL, and vehicle–qs 1 g. The chemicals were accurately weighed, transferred to an agate mortar, and then stepwise levigated with ethoxydiglycol and geometrically homogenized with the vehicle. The product was finally passed through a roll mill (Fagron, USA), collected, and stored in white airless and plunger packing (Emphasys, Brazil).

Products characterization

The mean particle size and zeta potential of the products were measured by laser dynamic light scattering (DLS; Microtrac, USA). The analysis was performed at a measuring angle of 180° at 25 °C using samples appropriately diluted with MeOH. Values were reported as the average particle size (diameter, μm) and polydispersity index (n = 3).

Standard and sample solutions

Accurately weighed amounts (analytical digital balance AY220; Shimadzu, Japan) of the standards were dissolved and diluted in appropriate solvents (T and P = EtOH, E2 and E3 = MeOH) to obtain work solutions with the following concentrations: T = 20 μg mL⁻¹, P = 50 μg mL⁻¹, E2 = 20 μg mL⁻¹, E3 = 80 μg mL⁻¹. T standard was dried in vacuum over phosphorus pentoxide for 4 h before use. The transdermal emulsions were diluted in the same manner and at the same concentrations as their respective standards.

Quantification of hormones–HPLC

HPLC analyses were performed on a qualified and calibrated chromatography system (Young Lin, Korea) composed of a quaternary pump (YL 9110), a photodiode array detector (YL 9160), an automatic injector (YL 9150), a column compartment (YL 9130), and a software controller (Clarity). Chromatographic separation was achieved using octadecylsilane (L1/C18) columns; 250 × 4.6 mm, 5 μm particle size (for P) and 125 × 4.6 mm, 5 μm particle size (for T, E2 and E3) (Phenomenex, USA). The columns were connected with a pre-column (C18, 4.0 × 3.0 mm, 5 μm) from the same manufacturer.

The major focus of our study was the development of an eco-friendly method, specific for each product/emulsion examined. HPLC was used to separate the active ingredients from the emulsions matrix (the multi-ingredient vehicle Pentravan® and ethoxydiglycol). After initial trial measurements, we were able to obtain an optimal chromatographic experimental design, which offered a short run time and use of minimal starting reagents. Thus, a 2³ central composite design (2³ CCD) of three factors, two main levels, six axial levels, and triplicate in central point, totaling 17 experiments, was employed as a way to assess favorable conditions for the different methods in a non-empirical manner. The different variables (factors) considered and their levels are listed in Table 1. Other experimental conditions, including the volume of injection (20 μL), mobile phase (flow rate = 1.2 mL min⁻¹), and wavelengths (241 nm for T; 254 nm for P; 205 nm for E2 and E3), were maintained constant. The experiments were performed in random sequence; therefore, any distortion of the statistical results was avoided.

Validation

After separately determining the most suitable methods for each product/emulsion, validation tests were performed according to the International Conference on Harmonization¹⁰ and the Brazilian National Institute of Metrology, Standardization and Industrial Quality¹¹ guidelines, as conventionally performed by our group.^{12,13}

Specificity

The specificity was determined using student's t-test for the comparison of hormone quantification (mean values) from the analyte in the presence and absence of the matrix (Pentravan® + ethoxydiglycol). The acceptance criterion was defined as a percentage of discrepancy between the results, lower than 2%. In addition, the specificity of the method was obtained through comparison of standard chromatograms with and without the matrix.

Linearity

The test was performed to assess the linear relationship between the concentration of the analyte and the obtained areas. This was performed by plotting three standard curves, each constructed by the five different hormone concentrations of the work solutions (i.e., 14, 17, 20, 23, and 26 μg mL⁻¹ for T in T_{emuls}; 35, 42.5, 50, 57.5, and 65 μg mL⁻¹ for P in P_{emuls}; 14, 17, 20, 23, and 26 μg mL⁻¹ for E2 in E_{emuls}, and Biest; 56, 68, 80, 92, and 104 μg mL⁻¹ for E3 in Biest). For this purpose, the data for each concentration range after fitting by the ordinary least-squares method were statistically evaluated; this method accounted for homoscedasticity (Cochran's test), residues' normality (Shapiro–Wilk test), and the lack of fit test (analysis of variance, ANOVA).

Limits of Detection and Quantification

The limit of detection (LOD) and limit of quantification (LOQ)

Table 1. 2³ CCD for human sexual steroids transdermal emulsions methods optimization

Issue	Matrix X			Vectors y*									
				T _{emuls}		P _{emuls}		E _{emuls}		E2-Biest		E3-Biest	
	X ₁	X ₂	X ₃	Assay	t _R	Assay	t _R	Assay	t _R	Assay	t _R	Assay	t _R
1	-1	-1	-1	99.33	6.4	104.00	11.6	102.43	13.3	88.53	23.6	96.57	4.0
2	1	-1	-1	95.56	3.2	97.35	7.0	105.47	11.9	nd	nd	98.40	14.5
3	-1	1	-1	99.55	6.4	98.86	11.8	99.05	13.1	101.50	23.5	101.07	3.9
4	1	1	-1	98.93	3.3	95.25	7.7	102.64	11.9	nd	nd	97.80	14.2
5	-1	-1	1	96.95	4.9	99.05	9.7	102.28	12.5	103.21	14.9	99.88	3.2
6	1	-1	1	98.67	2.8	103.33	5.9	112.44	12.0	nd	nd	101.44	8.9
7	-1	1	1	100.20	4.9	93.48	9.4	106.04	12.7	99.51	13.9	98.06	2.9
8	1	1	1	100.52	2.8	96.61	5.7	107.17	12.4	nd	nd	45.09	8.4
9	-1.68	0	0	98.91	7.3	104.57	12.6	104.20	11.2	115.01	12.7	nq	2.7
10	1.68	0	0	100.17	2.8	96.40	6.1	102.31	12.1	nd	nd	94.43	16.8
11	0	-1.68	0	95.48	4.0	96.48	8.5	102.51	10.9	87.94	41.2	84.68	5.7
12	0	1.68	0	101.70	4.0	98.93	8.2	107.22	9.8	103.02	41.1	102.99	5.7
13	0	0	-1.68	98.35	4.9	96.55	8.1	109.33	10.2	nd	nd	nq	8.3
14	0	0	1.68	99.64	3.4	95.07	6.9	103.98	12.1	100.44	24.7	99.04	4.1
15	0	0	0	100.97	4.0	107.45	7.5	102.55	7.6	99.78	40.0	101.64	5.6
16	0	0	0	100.65	4.0	105.65	7.5	101.89	7.6	99.89	40.0	101.10	5.6
17	0	0	0	99.90	4.0	106.69	7.5	100.75	7.6	100.41	40.0	99.63	5.6

*Assay as % and t_R (retention time) as min. nd: not detectable. nq: not quantifiable. All experiments provided good factor capacity of the chromatographic column (>2.0), symmetry of the analytical peak (>1.0) and column efficiency (number of theoretical plates/meter > 500).

For the experimental design, the levels of the factor were defined as (theoretical level outside the parenthesis and real values inside them) ($\alpha = [2k]^{1/4}$, and $k = 3$; then $\alpha = 1.68$):

X₁ = EtOH (%) in mobile phase [T_{emuls}: -1.68 (47), -1 (50), 0 (55), 1 (60), 1.68 (63); P_{emuls}: -1.68 (52), -1 (55), 0 (60), 1 (65), 1.68 (68); E_{emuls}: -1.68 (43.4), -1 (44), 0 (45), 1 (46), 1.68 (46.6); Biest: -1.68 (22), -1 (25), 0 (30), 1 (35), 1.68 (38)].

X₂ = ultrasound dissolution of sample (min) [T_{emuls}: -1.68 (3), -1 (10), 0 (20), 1 (30), 1.68 (37); P_{emuls}: -1.68 (3), -1 (10), 0 (20), 1 (30), 1.68 (37); E_{emuls}: -1.68 (22), -1 (25), 0 (30), 1 (35), 1.68 (38); Biest: -1.68 (3), -1 (10), 0 (20), 1 (30), 1.68 (37)].

X₃ = Column temperature (°C) [T_{emuls}: -1.68 (18), -1 (25), 0 (35), 1 (45), 1.68 (52); P_{emuls}: -1.68 (18), -1 (25), 0 (35), 1 (45), 1.68 (52); E_{emuls}: -1.68 (18), -1 (25), 0 (35), 1 (45), 1.68 (52); Biest: -1.68 (18), -1 (25), 0 (35), 1 (45), 8 (52)].

were determined from three standard calibration curves and were calculated as shown in Eq. (1) and (2), respectively:

$$LOD = S \frac{3}{a} \quad (1)$$

$$LOQ = S \frac{10}{a} \quad (2)$$

where a is the slope of the calibration curve, and S is the standard deviation of the y-intercept. The LOD and LOQ were confirmed by the analysis of chromatograms generated by injecting solutions in their respective limit concentrations.

Precision

The test was designed to assess the degree of dispersion among the series of measurements obtained by the same analyst (intra-assay precision, repeatability) and between two analysts and two days (within-lab variations, intermediate precision) for solutions of T at 20 µg mL⁻¹, P at 50 µg mL⁻¹, E2 at 20 µg mL⁻¹ (in E_{emuls} and Biest), and E3 at 80 µg mL⁻¹. Repeatability was determined by consecutively analyzing six replicates by a single analyst in a single day. Intermediate precision was also performed in six replicates, but in two days, by different analysts. An injection precision of <5 % relative to the standard deviation was considered appropriate.

Accuracy

Accuracy measurements were performed by the same analyst by injecting the chromatographic samples to which the matrix was added (at the same concentrations levels performed for the linearity

test ($n = 3$ for each concentration level)). The result was expressed as a percentage of recovery, compared with the analytical curve obtained from linearity.

Robustness

To evaluate the significance that the deliberate variations play in each chosen factor (X₁: EtOH percentage in the mobile phase; X₂: column oven temperature; X₃: mobile phase flow rate), a complete experimental design with eight experiments (2³) and triplicate in the central point was conducted, with a total of 11 experiments performed in random order. The factors and their levels studied are listed in Table 3.

From the results of each experiment, the coefficients for determining the statistical model of prediction were calculated according to Eq 3:

$$b = (X^T X)^{-1} X^T y \quad (3)$$

where b is the matrix of model coefficients, and X and y are the X matrix and vector y , respectively. To derive the equation of the fitted model, the standard errors of the coefficients were calculated using Eq. 4:

$$\varepsilon(b) = \sqrt{(X^T X)^{-1} \cdot \sigma^2} \quad (4)$$

where $\varepsilon(b)$ is the matrix whose main diagonal represents the standard errors of the model estimators (b_i), and σ^2 is the population variance of the experiments, which can be estimated as s^2 using the center point replicates from Eq. 5:

$$s^2 = \frac{\sum (x_i - \bar{x})^2}{(n-1)} \quad (5)$$

Effects were calculated in the matrix by the product $X'y$, where y is a column vector containing the average results of the assay.

To estimate the standard error of an effect, the square root of the value obtained in Eq. 6 was used, and the standard error of the mean was estimated using the square root of the value obtained in Eq. 7:

$$\hat{V}_{effect} = \left(\frac{s^2}{2} \right) \quad (6)$$

$$\hat{V}_{mean} = \left(\frac{s^2}{n} \right) \quad (7)$$

With the estimated standard errors, it was possible to achieve confidence intervals for the values of effects using the student's t distribution with 95% confidence (Eq. 8):

$$\hat{\eta} - t_v \cdot S_{effect} < \eta < \hat{\eta} + t_v \cdot S_{effect} \quad (8)$$

where η is the true value of an effect (population value), $\hat{\eta}$ represents the value obtained from the tests performed on the experiment, t_v is the value from the student's distribution, and S_{effect} is the standard error of an effect.

Selection of receptor medium for *in vitro* release tests

The validated methods were then employed for *in vitro* drug release tests, where biorelevant media were prepared according to the procedure described by Baert et al.¹⁴ To select the most suitable medium for each product/emulsion, the following compositions were used: 0.01 M phosphate buffered saline, pH 7.4 (NaCl–138.0 mM; KCl–2.7 mM; KH_2PO_4 –1.43 mM; Na_2HPO_4 –8.57 mM); artificial human sweat (NaCl–49.96 mM; CaCl_2 –0.15 mM; MgSO_4 –1.0 mM; KH_2PO_4 –7.5 mM); simulated body fluid (NaCl–136.8 mM; KCl–3.0 mM; CaCl_2 –2.5 mM; MgCl_2 –1.5 mM; Na_2SO_4 –0.5 mM; NaHCO_3 –4.2 mM; KH_2PO_4 –1.0 mM). The solubility of these compositions was also determined with additional measurements using bovine serum albumin (BSA) or hydroxypropyl- β -cyclodextrin (HPBCD) as the medium, resulting in a total of nine different media samples. A standard receptor medium consisting of MeOH and H_2O (70:30, v/v) was also prepared for reference. Aliquots (10 mg) of each hormone were accurately weighed in 10 individual 10 mL glass tubes, and each receptor medium was added to volume in its respective tube. Subsequently, the tubes were shaken for 10 min, sonicated for a further 30 min, and then left in a water bath (32 °C) overnight (12 h). The tubes were then centrifuged at 20,000 $\times g$, and the clear supernatant was transferred into glass HPLC vials for quantification.

In vitro release

In vitro release tests were performed in 7-mL static vertical diffusion cells with automatic sampling (Microette Plus®, Hanson Research, USA). The donor compartment contained the hormone formulations ($n = 6$ for each formulation), and the receptor compartment was filled with the respective receptor medium, ensuring that air under the artificial membrane was completely eliminated. Polysulfone membrane disc filters, 25 mm diameter (Tuffryn®, Pall Corporation, USA), were rinsed to remove any additives that prevented drug release from the formulations and were positioned between the cell compartments. An infinite dose (300 mg) of each formulation was applied to the membrane surface using a calibrated positive displacement pipette Pos-D MR-110 (Rainin, USA), which prevented solvent evaporation

and reduced any compositional change. The emulsions were then carefully spread to achieve complete uniform coverage, with a diffusion area of 1.86 cm², with the compartments held together using a clamp. The receptor medium was continuously mixed using a magnetic stirring bar (300 rpm, 32 ± 2 °C during the entire measurement), except during the sample collecting period. Aliquots (1 mL) were withdrawn at regular time intervals (0.5, 1, 2, 3, 4, 8, 12, 16, 20, and 24 h), collected into HPLC vials, and immediately replaced with the receptor medium at the same temperature. The hormone concentrations were correspondingly corrected for the replenishments.

The diffused quantity of the drug ($Q_{real,t}$) in the time t was calculated using Eq. 9:

$$Q_{real,t} = C_{measured,t} \cdot V_r \cdot V_a \cdot \sum^{n-1} C_a \quad (9)$$

where $C_{measured,t}$ is the concentration measured at sampling time t , V_r is the volume of the diffusion cell, V_a is the aliquot volume, n is the number of the sampling at time t , and C_a is the concentration of the aliquot.

Mathematical models were applied to determine the diffusion kinetics: cumulative amounts of drug diffusion per unit area ($\mu\text{g cm}^{-2}$) were plotted against time (h) for zero-order kinetics; cumulative amounts of drug diffusion per unit area ($\mu\text{g cm}^{-2}$) were plotted against the square root of time ($\sqrt{h}$) for the Higuchi model; log of the cumulative amounts of drug diffusion per unit area ($\log \mu\text{g cm}^{-2}$) was plotted against time (h) for first-order kinetics. The coefficient of determination (R^2) was calculated, and those with a value >0.99 were considered linear. Steady-state diffusion flux (J_s) was then determined from the linear slope of the cumulative amount of hormone *versus* time curves. Here the lag time (T_L) represented the time required to achieve a steady-state flux.

RESULTS AND DISCUSSION

Products manipulation and characterization

The products were compounded following a standard protocol developed in our laboratory with the PentraVan® vehicle, which was readily available in its liposomal form. Therefore, the inclusion of drugs into the vesicles could be achieved by a simple roll-mill technique. The characterization of particle size was performed, and the results (in μm) were 1.654 for T_{emuls} , 1.008 for P_{emuls} , and 1.672 for Biest, which indicated that the products were small and comparable in size. The polydispersity indexes of the vesicles were 5.55 for T_{emuls} , 1.55 for P_{emuls} , and 14.20 for Biest, indicating relatively homogeneous population of vesicles. These results were encouraging as small particles are particularly suitable in transdermal applications, where decreasing vesicles' particle size has been reported to increase the penetration of encapsulated drugs into deeper skin layers.¹⁵

Method development

The PentraVan® vehicle and the corresponding formulations were completely soluble in MeOH and EtOH, with the exception of E3-containing formulation, which was only soluble in MeOH. However, because of the health risks linked to this solvent (e.g., blindness) and high toxicity (in severe cases¹⁶ owing to the formation of formic acid *in vivo*¹⁷), we chose to work with MeOH only in formulations containing E3. In efforts to make the process environmentally benign, the less hazardous EtOH was used as the standard solvent for the formation of mobile phases and solubilization of samples.¹⁸ The following conditions were defined for the initial chromatographic analysis of transdermal bioidentical hormones: column and pre-column C18;

injection volume = 20 μL ; mobile phase (flow rate = 1.2 mL min^{-1}); detection by spectrophotometric scanning between wavelengths of 190 and 900 nm (in this case, maximum responses were obtained at 241 nm for T, 254 nm for P, and 205 nm for E2 and E3). A mixture of EtOH and H_2O (50:50, v/v) was used as an initial trial mobile phase because of its intermediate concentration of organic carbon. However, the preliminary chromatographic runs showed that this was not suitable because no chromatographic peak could be identified up to 30 min of analysis. This time duration was selected to make the analysis cost-effective and feasible in routine laboratory quality control. A 2^3 CCD was used to screen conditions that were optimal, eco-friendly, and applicable to a laboratory routine, and the results corresponding to these measurements are listed in Table 1 (prior to this design, pilot trials were conducted to define the range of variation for each parameter). Both parameters that gave optimal responses were chosen as the ideal condition, resulting in a method that provided good recovery without being time-consuming. However, the method for Biest was performed with increased time duration because of the difference in the properties of the formulation: E3 eluted rapidly and E2 exhibited a higher retention on the column. Nevertheless, optimal and environmentally friendly methods were successfully developed for all formulations studied using only water and ethanol as the mobile phase, in accordance with the principles of green chemistry. The final conditions for each formulation are listed in Table 2.

Validation

After development and optimization, the methods were validated. For linearity, calibration plots ($x = \mu\text{g mL}^{-1}$, $y = \text{mV}$) of the hormones measured by the proposed methods were constructed ($n = 3$, independent replicates). By fitting the samples' data from Table

3, the regression of the analytical curve was found to have no lack of fit (with 95% confidence). In addition, normality in the residues (Shapiro–Wilk test) and homoscedasticity (Cochran test) were applied to verify our model fit. Indeed, the normality showed p -values >0.05 , and the Cochran's C values were lower than the critical value, which indicated no violation of the assumptions; therefore, the ANOVA was considered valid.

The LOD (in $\mu\text{g mL}^{-1}$) were 0.45 for T, 1.68 for P, 0.64 for E2 in E_{emuls} , 3.18 for E2 in Biest, and 6.15 for E3 in Biest, and the corresponding LOQ (in $\mu\text{g mL}^{-1}$) were 1.52 for T, 5.62 for P, 2.15 for E2 in E_{emuls} , 10.60 for E2 for Biest, and 20.51 for E3 in Biest. These values were extremely imperative for our diffusion experiments, providing the quantification of the drug passing through the membrane in receptor media. Furthermore, injections of hormone solutions in their respective limited concentrations confirmed this observation.

The specificity was determined as a percentage of discrepancy lower than 2% by comparing the chromatographic areas of the analyte and analyte + matrix (the multi-ingredient vehicle Pentravan® and ethoxydiglycol, which represent possible interfering agents for the analyses). For T_{emuls} , these values were 1611695.41 ± 17726.02 and 1582838.27 ± 24417.39 ($\%_{\text{discrepancy}} = 1.79$); for P_{emuls} , 1866737.33 ± 9748.29 and 1861131.64 ± 7652.98 ($\%_{\text{discrepancy}} = 0.30$); for E_{emuls} , 1577383.33 ± 55672.35 and 1607322.46 ± 12144.64 ($\%_{\text{discrepancy}} = 1.90$); for E2-Biest, 1309365.14 ± 7208.50 and 1316888.06 ± 4921.52 ($\%_{\text{discrepancy}} = 0.57$); for E3-Biest, 5057112.67 ± 55687.05 and 4960697.67 ± 55169.99 ($\%_{\text{discrepancy}} = 1.94$). The chromatograms obtained (Figure S1) also demonstrated that indeed no matrix interference was observed in the analysis.

For precision, the coefficients of variation (in %) were found to be intra-day, first day ($n = 12$, 6 for each analyst) = 2.33 for T_{emuls} , 1.22 for P_{emuls} , 1.08 for E_{emuls} , 1.69 for E2-Biest, and 0.44 for E3-Biest;

Table 2. Final chromatographical conditions for human sexual steroids transdermal emulsions, determined after optimization

Formulation	Diluent	Work concentration ($\mu\text{g mL}^{-1}$)	Mobile phase	Flow rate (mL min^{-1})	Column	Oven temperature ($^{\circ}\text{C}$)	UV detection (nm)
T_{emuls}	EtOH	20 (20 min in US)	EtOH:H ₂ O (63:37, v/v)	1.2	C18 125 $\times$ 4.6 mm	35	241
P_{emuls}	EtOH	50 (10 min in US)	EtOH:H ₂ O (65:35, v/v);	1.2	C18 250 $\times$ 4.6 mm	45	254
E_{emuls}	EtOH	20 (30 min in US)	EtOH:H ₂ O (45:55, v/v)	1.2	C18 250 $\times$ 4.6 mm	25	205
Biest	MeOH	20 (E2) 80 (E3) (30 min in US)	EtOH:H ₂ O (35:65, v/v)	1.2	C18 125 $\times$ 4.6 mm	45	205

T_{emuls} : emulsion containing testosterone; P_{emuls} : emulsion containing progesterone; E_{emuls} : emulsion containing estradiol; Biest: emulsion containing estradiol and estriol; EtOH: ethanol; MeOH: methanol; H₂O: ultrapure water; UV: ultraviolet.

Table 3. Results from the linearity study and comparison of the diluents used

Formulation	Slope	Intercept	ANOVA ^d	Normality ^e	Homoscedasticity ^f
T_{emuls} (MeOH) ^a	42429 (± 859)	16939 (± 17585)	0.105	0.945	0.613
T_{emuls} (R_M) ^a	42715 (± 1010)	15787 (± 20625)	0.120	0.444	0.383
P_{emuls} (MeOH) ^b	48004 (± 1333)	-110741 (± 69288)	0.102	0.131	0.345
P_{emuls} (R_M) ^b	47559 (± 964)	-126471 (± 50105)	0.163	0.091	0.532
E_{emuls} (MeOH) ^a	82613 (± 1837)	14027 (± 37703)	0.133	0.470	0.351
E_{emuls} (R_M) ^a	76937 (± 1338)	83053 (± 27450)	0.712	0.500	0.393
E2-Biest (MeOH) ^a	68157 (± 1102)	-39961 (± 22667)	0.705	0.658	0.408
E2-Biest (R_M) ^a	69845 (± 1647)	-64646 (± 33828)	0.390	0.589	0.355
E3-Biest (MeOH) ^c	58308 (± 921)	68722 (± 75818)	0.213	0.682	0.478
E3-Biest (R_M) ^c	59722 (± 1001)	-68482 (± 82377)	0.118	0.632	0.607

$n=3$ (genuine replicates). MeOH: methanol. R_M : receptor medium. Range ($\mu\text{g mL}^{-1}$): ^a(14–26); ^b(35–65); ^c(56–104); ^dANOVA p -value, significance level $p < 0.05$; ^eResidue normality test p -value (Shapiro–Wilk test), significance level $p < 0.05$; ^fResidue homogeneity test (Cochran test), critical value = 0.684.

intra-day, second day ($n=12$, 6 for each analyst) = 1.29 for T_{emuls} ; 1.93 for P_{emuls} , 4.13 for E_{emuls} , 2.01 for E2-Biest, and 0.55 for E3-Biest; inter-days ($n=24$) = 2.24 for T_{emuls} , 1.69 for P_{emuls} , 3.03 for E_{emuls} , 3.42 for E2-Biest, and 2.72 for E3-Biest.

For accuracy, the average recoveries of the active ingredients (in %) were 98.30 for T, 98.03 for P, 98.03 for E2, 98.18 for E2-Biest, and 98.09 for E3-Biest, thus meeting the acceptance criteria of 98.0%–102.0% for this parameter.

For robustness, a complete experimental design with eight experiments (2^3) and triplicate in the central point was conducted, with a total of 11 experiments performed in random order (Table 4). The only variation that played a significant role in the assay was the column compartment temperature (column oven) for T_{emuls} and E_{emuls} . Interestingly, the temperature had a positive effect on the T_{emuls} and a negative effect on P_{emuls} . No other factors affected the recovery of the actives, and the methods for E_{emuls} and Biest displayed no interference in their relevant assay. These data suggest that the methods are robust, even for T_{emuls} and E_{emuls} , once the column temperature has been adjusted and controlled with precision by the chromatographic equipment.

Receptor medium for *in vitro* drug release study

Once the methods had been assessed, the next stage of our study was to use the formulations for *in vitro* drug release. Initial investigations were performed to examine the solubility of the formulations

in the receptor medium (as shown in Table 5). In general, we observed low solubility across the different compositions, except for the solution that consisted of MeOH and H_2O . However, in practice, their use was not feasible as they were not biocompatible with the receptor medium for establishing an *in vitro*–*in vivo* relationship.¹⁴ As a concentration of 50 g L^{-1} is quite often accepted as the mean albumin concentrations in human serum,¹⁹ 5% of BSA was added in all biorelevant media. As an alternative, 0.5% of HPBCD was also added.

Importantly, the solubility of these media was lower than in MeOH: H_2O (70:30, v/v); however, it was sufficiently high to ensure that it was not rate limiting for our permeation studies. The linearity of the method was also confirmed as the hormones were diluted in the receptor medium, rather than their diluents. These results shown in Table 3 confirm that the method can be used for both quality control of semi-solid products and analysis of *in vitro* drug release.

In vitro drug release

The *in vitro* drug release measurement is currently widespread as a method to assess the release of drugs from semi-solid systems, thereby foreseeing the percutaneous absorption of topically applied, but systemically active, transdermal formulations.²⁰ Nevertheless, the applications of the *in vitro* drug release are not limited as they also permit the characterization and differentiation of formulations—to assess product quality and comparatively evaluate innovative products over

Table 4. Factors, levels, contrast coefficients matrix for the experimental design conducted to the robustness study and the respective calculated effects.

Experiment	Matrix X								Vectors y – Assay (%)				
	Mean	X ₁	X ₂	X ₃	X ₁₂	X ₁₃	X ₂₃	X ₁₂₃	T _{emuls}	P _{emuls}	E _{emuls}	E2-Biest	E3-Biest
1	1	–1	–1	–1	1	1	1	–1	99.33	103.98	102.43	87.29	97.40
2	1	1	–1	–1	–1	–1	1	1	95.56	98.38	105.17	0.00	98.40
3	1	–1	1	–1	–1	1	–1	1	99.55	98.86	99.05	101.50	101.07
4	1	1	1	–1	1	–1	–1	–1	98.93	96.39	102.64	0.00	97.80
5	1	–1	–1	1	1	–1	–1	1	96.95	99.03	102.28	103.21	99.88
6	1	1	–1	1	–1	1	–1	–1	98.67	103.31	112.44	0.00	101.44
7	1	–1	1	1	–1	–1	1	–1	100.20	93.48	106.04	97.02	96.43
8	1	1	1	1	1	1	1	1	100.52	96.61	107.17	0.00	96.42
9	1	0	0	0	0	0	0	0	100.97	107.45	112.55	95.55	101.64
10	1	0	0	0	0	0	0	0	100.65	105.65	107.89	104.54	101.10
11	1	0	0	0	0	0	0	0	99.90	106.69	107.75	100.41	99.63
Effects on Assay													
				T _{emuls}		P _{emuls}		E _{emuls}		E2-Biest		E3-Biest	
Mean				99.20 ± 0.01		100.90 ± 0.01		105.95 ± 0.82		62.68 ± 1.36		99.20 ± 0.31	
Principal effects													
X ₁ : EtOH (%) in mobile phase				–0.59 ± 0.03		–0.16 ± 0.03		4.41 ± 1.93		–97.26 ± 3.18		–0.18 ± 0.74	
X ₂ : Column temperature (°C)				2.17 ± 0.03		–4.84 ± 0.03		–1.86 ± 1.93		2.00 ± 3.18		–1.35 ± 0.74	
X ₃ : Flow rate (mL min ^{–1})				0.74 ± 0.03		–1.30 ± 0.03		4.66 ± 1.93		2.86 ± 3.18		–0.13 ± 0.74	
Two-factors interactions													
X ₁₂				0.44 ± 0.03		0.49 ± 0.03		–2.04 ± 1.93		–2.00 ± 3.18		–1.46 ± 0.74	
X ₁₃				1.61 ± 0.03		3.87 ± 0.03		1.24 ± 1.93		–2.86 ± 3.18		0.96 ± 0.74	
X ₂₃				0.38 ± 0.03		–1.29 ± 0.03		1.10 ± 1.93		–5.10 ± 3.18		–2.88 ± 0.74	
Three-factors interactions													
X ₁₂₃				–1.13 ± 0.03		–1.07 ± 0.03		–2.47 ± 1.93		5.10 ± 3.18		0.67 ± 0.74	
t _v · S _{effect}				1.67		2.74		8.31		13.69		3.17	

All experiments provided good factor capacity of the chromatographic column (>2.0), symmetry of the analytical peak (>1.0) and column efficiency (number of theoretical plates/meter > 500).

Shadowed columns represent statistically significant effects ($p < 0.05$).

X_1 = EtOH (%) in mobile phase [T_{emuls} : –1 (61), 0 (63), 1 (65); P_{emuls} : –1 (63), 0 (65), 1 (67); E_{emuls} : –1 (43), 0 (45), 1 (47); Biest: –1 (33), 0 (35), 1 (37)].

X_2 = Column temperature (°C) [T_{emuls} : –1 (33), 0 (35), 1 (37); P_{emuls} : –1 (43), 0 (45), 1 (47); E_{emuls} : –1 (23), 0 (25), 1 (27); Biest: –1 (42), 0 (45), 1 (47)].

X_3 = Mobile phase flow rate (mL min^{–1}) [T_{emuls} : –1 (1.0), 0 (1.2), 1 (1.4); P_{emuls} : –1 (1.0), 0 (1.2), 1 (1.4); E_{emuls} : –1 (1.0), 0 (1.2), 1 (1.4); Biest: –1 (1.0), 0 (1.2), 1 (1.4)].

Table 5. Results of the maximum solubility of the hormones in the receptor media

Receptor media	Average solubility ($\mu\text{g mL}^{-1}$, $n = 3$)			
	Testosterone	Progesterone	17- β -Estradiol	Estriol
PBS	130.95	30.52	20.64	190.86
PBS + 5% BSA	530.40	150.85	10.77	270.20
PBS + 0.5% HPBCD	490.86	360.49	60.82	560.40
SS	200.05	80.36	10.69	220.15
SS + 5% BSA	540.49	210.12	30.57	300.27
SS + 0.5% HPBCD	560.08	270.84	20.98	550.87
SBF	190.97	60.80	10.67	190.49
SBF + 5% BSA	430.35	170.32	20.15	230.89
BFS + 0.5% HPBCD	290.86	70.71	10.30	190.30
MeOH : H ₂ O (70:30, v/v)	962.74	905.78	664.78	815.74

PBS: 0.01 M phosphate buffered saline, pH 7.4. SS: artificial human sweat. SBF: simulated body fluid. BSA: bovine serum albumin. HPBCD: hydroxypropyl- β -cyclodextrin. MeOH: methanol. H₂O: purified water. Shadowed columns represent the higher solubilities.

the already available equivalents in the market.²⁰ For instance, changes in the drugs' structural characteristics or thermodynamic properties can lead to varying release rates, which may affect the permeation and bioavailability of the transdermal product.²¹ The method is also beneficial for reducing the overall time for conducting *in vitro* models before *ex vivo* or *in vivo* tests. Given its importance and potential, it is expected that such a process will be a leading contender in future medical diagnostics, e.g., in pharmacopeia testing according to the United States Pharmacopeia²²

The experiments described herein were performed using membranes that do not limit the flux rate from the donor to receptor compartments. These membranes are considerably useful for the development and assessment of drug release kinetics from semi-solid formulations as they permit the determination of the maximum liberation rate of the active substances from an emulsion matrix.¹⁹ Furthermore, the synthetic membrane model is not influenced by the pH of the formulation, partition coefficient between the drug or the skin, or inherent factors of the source of skin samples, including sex, age, and site of skin excised.²³ The use of an artificial membrane is also justified as it minimizes the number of experimental variables for gaining an in-depth understanding of hormone release.

Departing from this scenario, the *in vitro* release profiles of the drugs studied within the PentraVan® vehicle were constructed, and they are shown in Figure 2. From the obtained results, it is apparent that the release profiles were not linear, with the exception of T_{emuls}, which suggests that only its release follows zero-order kinetics. Indeed, the data available in Table 6 confirms this finding as T_{emuls} displayed $R^2 > 0.99$ for the zero-order kinetics, while remaining emulsions fitted best for the pseudo-first-order model, also known as the Higuchi's model. Thus, the rate of T_{emuls} release does not increase when the active substance concentration is increased, i.e., the amount of testosterone released is not proportional to time. In contrast, the formulations that follow the Higuchi's model confirm that the release mechanism of these drugs is via diffusion based on Fick's law and is dependent on the square root of time, which is typical in matrix-type products.

The total amounts released after the 24-h experiment were 181.18 $\mu\text{g cm}^{-2}$ for T, 135.75 $\mu\text{g cm}^{-2}$ for P, 18.97 $\mu\text{g cm}^{-2}$ for E2 in E_{emuls}, 25.51 $\mu\text{g cm}^{-2}$ for E2 in Biest, and 62.05 $\mu\text{g cm}^{-2}$ for E3 in Biest. Notably, the release profile of T_{emuls} was observed to be fairly linear, qualitatively. Meanwhile, the other formulations exhibited a gradual

Figure 2. Release profiles of human sexual hormones. Results are presented as mean $\pm$ standard deviations ($n = 6$)

rise until a discrete peak was detected, followed by a relatively sustained release.

Moreover, the *in vitro* release data was complemented with the determination of flux (J_s , $\mu\text{g cm}^{-2} \text{h}^{-1}$) and lag time (L_p , h), parameters presented in Table 6. Amongst the formulations, P_{emuls} exhibited the highest drug release, which can be attributed to its high solubility within the cream matrix, thereby facilitating the release from the three-dimensional net of the emulsion into the receptor medium. In addition, the release rate or the steady-state flux is formulation-specific and can be used to monitor product quality.

Interestingly, the lag times were medially all close to 16.8 min (12–21.6), except for the E2 in the Biest formulation, which was significantly low (0.6 min). This could presumably be attributed to the higher concentration of E2 compared with E_{emuls} and also an unresolved synergistic effect between E2 and E3 within the Biest formulation.

CONCLUSION

In conclusion, we successfully developed and validated novel HPLC methods that are facile and eco-friendly for transdermal emulsions containing human sexual steroids (testosterone, progesterone,

Table 6. Mathematical modeling of the *in vitro* drug release kinetics

Drug	Mathematical Model	Equation	R^2	J_s ($\mu\text{g cm}^{-2}\text{h}^{-1}$)	T_L (h)
T (T_{emuls})	Zero-order	$y = 7.36x + 2.65$	0.999	7.36	0.36
	Higuchi	$y = 41.14x - 39.84$	0.967		
	First-order	$y = 0.06x + 1.09$	0.832		
P (P_{emuls})	Zero-order	$y = 5.14x + 18.25$	0.983	29.36	0.20
	Higuchi	$y = 29.36x - 13.10$	0.990		
	First-order	$y = 0.04x + 1.34$	0.819		
E2 (E_{emuls})	Zero-order	$y = 0.66x + 3.70$	0.981	4.22	0.22
	Higuchi	$y = 4.21x - 1.99$	0.997		
	First-order	$y = 0.03x + 0.67$	0.884		
E2 (Biest)	Zero-order	$y = 0.89x + 5.52$	0.957	5.18	0.01
	Higuchi	$y = 5.18x - 0.14$	0.997		
	First-order	$y = 0.03x + 0.76$	0.789		
E3 (Biest)	Zero-order	$y = 2.47x + 6.99$	0.972	14.24	0.35
	Higuchi	$y = 14.2x - 8.39$	0.999		
	First-order	$y = 0.04x + 0.90$	0.737		

J_s : steady-state flux. T_L : lag time. Results expressed as mean of six replicates. T: testosterone; P: progesterone; E2: 17- β -estradiol; E3: estriol.

estradiol, or estradiol and estriol). Furthermore, the physiological receptor media for these formulations were successfully determined for *in vitro* drug release. Moreover, drug release measurements were performed to obtain optimal release rates of formulations. From this, we discovered that the vehicle (Pentravan®) exhibited high releases rates of incorporated hormones, proving to be an optimal option for transdermal route.

SUPPLEMENTARY MATERIAL

The sample chromatograms are available free of charge at <http://quimicanova.s bq.org.br>.

ACKNOWLEDGMENTS

The authors would like to thank the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the scholarship granted to Hudson Polonini and the Fagron team for the generous donation of reagents.

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