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Method Development and Validation of Stability Indicating RP-HPLC Method for the Determination of Female Hormones in Hormone Concentrates Creams

Pradeep Gautam* and Troy Purvis

Humco Research and Development Laboratory, 201 W. 5th St. Suite 1250, Austin, TX 78701, USA

Abstract

A rapid, novel, and efficient stability indicating reverse-phase high performance liquid chromatographic (RP-HPLC) analytical method was developed and validated for the determination of four female hormones: Progesterone, Estrone, Estradiol, and Estriol in Hormones Concentrate compounding bases. Separation was achieved with a RP- C18 Phenomenex® Gemini 150 × 4.6 mm C18 5µm column using a phosphate buffer pH 6.4 and acetonitrile. The developed HPLC method was validated with method validation components: linearity, accuracy, precision, intermediate precision, and robustness. Limit of detection (LOD) and limit of quantification (LOQ) were also obtained for all four hormones for this method. Photodiode array (PDA) detector was set up at 225 nm wavelength for the simultaneous analysis of all four peaks. Hormone concentrate samples, placebo, and hormone actives were subjected to stress conditions of hydrolysis (acid and base), oxidative, and thermal stress degradation. Standard solution stability was also performed. This female hormones HPLC method was validated as per the International Conference on Harmonization (ICH) guidelines. The proposed validated method was successfully used for the quantitative analysis of bulk, stability, and finished hormone concentrate and hormone compounding formulations.

Keywords: Female hormones; Hormone Concentrate Program (HCP); Method validation; Progesterone; Estrone; Estradiol; Estriol

Introduction

There are four main endogenous female steroid hormones that are responsible for the maintenance of the female sexual organs and control the development of the female secondary sexual characteristics. These hormones include three estrogens, Estrone (E1), Estradiol (E2), and Estriol (E3), as well as the progestogen Progesterone (P4). These chemicals serve important physiological roles in the female body (and the male body to a lesser extent) [1]. These chemicals are highly lipophilic compounds that can diffuse readily through the lipid membranes of animal cells, and once inside a cell, they can bind to receptor sites which activate and modulate the transcription of any number of genes that influence major changes in the whole individual [2].

Throughout the fertility cycle of a woman, she will experience these hormones in varying amounts and ratios according to which phase of her fertility cycle she is in. During pregnancy, for example, Estriol is the predominate estrogen that remains in a woman's unbound blood circulation, while Estrone becomes the dominate estrogen during menopause. Progesterone is the predominate progestogen that is involved in the menstrual cycle, and it supports pregnancy and the ability of the female to carry a baby to term [3]. All estrogens are synthesized in the body from androgen (male) steroid hormones by the action of aromatase enzymes, with the androgens Dehydroepiandrosterone, Testosterone, and Androstenedione becoming Estriol, Estradiol, and Estrone, respectively, upon metabolism by aromatase [4,5]. All hormones - estrogens, androgens, and progestogens alike - are important, therefore, in maintaining a healthy and sexually viable individual of either gender.

An imbalance of any one of the sex hormones can have a drastic negative effect on a person, so common diseases can arise from these imbalances. One, or more commonly a combination of several of these hormones can be used to treat female disease states that result from low hormone levels due to hypogonadism or age-induced menopause or surgical menopause. Hormone Replacement Therapy (HRT) supplements low estrogen levels, and therefore, serves an important role to alleviate uncomfortable symptoms like hot flashes or mood swings to more serious conditions like osteoporosis and dementia [3]. Additional Progesterone dosed as HRT reduces symptoms of severe PMS, treats certain types of infertility, and can support pregnancies in those that are high-risk, preventing miscarriage [6,7].

HRT is most commonly dosed via the transdermal route due to a few important factors. Poor oral bioavailability of hormones prevents some estrogen compounds from being dosed efficaciously in oral tablets or capsules. First-pass metabolism of hormones is prevented by transdermal application of HRT, and HRT offers a convenience factor that is not afforded by other administration routes. Suppositories can be messy and inconvenient, while buccal tablets can interfere with eating or speaking and are inappropriate in some instances. It is important for the physician to communicate which areas of the body have skin that is thin yet contains high vasculature for systemic transdermal HRT to be achieved. The excipient vehicle an important consideration when considering transdermal HRT. Since most transdermal HRT is prescribed as an extemporaneously compounded cream, the compounding base must have known absorption and penetration ability to carry the hormones across the dermal layers into the capillary

***Corresponding author:** Pradeep Gautam, Humco Research and Development Laboratory, 201 W. 5th St. Suite 1250, Austin, TX 78701, USA, Tel: 7378023159; Fax :5124747414; E-mail: pgautam@humco.com

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bed to enter circulation. Penetration enhancers must be present that allow for hormones to reach the circulation in a controlled manner and not all at one time such that undesirable effects result [8]. Overdosing of hormones in this way can cause a variety of adverse reactions from skin acne to migraine headaches, and overdosing over a over a long period of time cause liver damage, breast or uterine cancers, and blood thromboses [9].

Because sex hormones are very potent compounds where a small amount can elicit a large physiological response, and because high doses of hormones over extended periods can cause cancers, these compounds are listed as Hazardous Drugs by the United States Pharmacopoeia (USP). The USP has proposed guidance in USP <800> which mandate engineering controls to minimize the production of airborne particles of Hazardous Drugs [10]. Since compounded HRT is routinely formulated in compounding pharmacies, USP <800> requires these pharmacies to expend significant capital to build and install these engineering controls. Most pharmacies that compound HRT do not operate with enough profit margin to afford the expenses that accompany these build-outs, so other ways of minimizing employee exposure to airborne Hazardous Drug particulates have been explored. Incorporating hormone Hazardous Drugs in a concentrated form into a cream base would eliminate the possibility of hazardous airborne particles forming. These concentrates can be made such that aliquots of them can be weighed, mixed with, and diluted by either other hormone concentrates or additional non-hormone containing cream base to make a final prescription that contains any combination of hormone in any amount that could be possible for dispensing. If these concentrates were produced at a facility that follows cGMP, then exact potencies of these concentrates would be known and certified, reducing error that could be made by producing these concentrates on a small-scale pharmacy level. In any case, it is important to be able to analyze HRT or Hormone Concentrates to determine the potency of the hormones contained in them, so this manuscript delineates the method validation elements and corresponding acceptance criteria used to validate an HPLC method for the determination of female hormones in an HRT transdermal cream base.

Combination as well as individual female hormones in topical cream are extensively prescribed in the compounding pharmacy and so there is a great need of an accurate quantification method and simultaneous determination of all those hormones at the same run. There was a necessity of robust and stability-indicating method for the determination of female hormones compounded in a topical base. This manuscript describes a simple, specific, precise stability-indicating HPLC method for the determination of four female hormones (Progesterone, Estrone, Estradiol, and Estriol) in individual concentrate bases (Progesterone 40% w/w, Estrone 1% w/w, Estradiol 10% w/w, and Estriol 10% w/w). Chemical structures of each hormone are shown in Figures 1-4. The method was adequately developed and validated by general guidelines described in ICH guidelines in ICH publication Q2 (R1) Validation of Analytical Procedures [11].

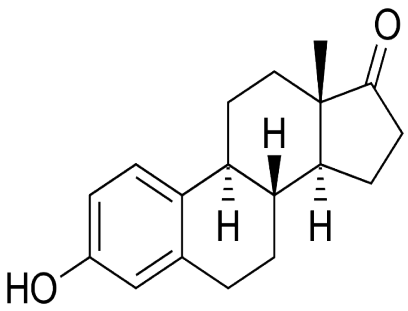

Figure: 1 Estrone (E1).

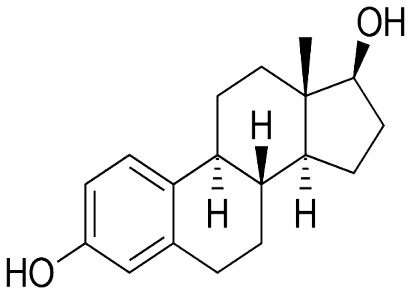

Figure 2: Estradiol (E2).

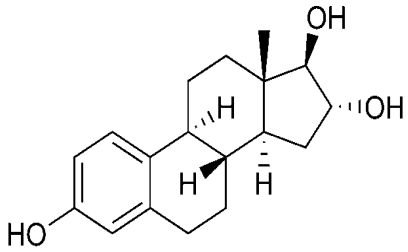

Figure 3: Estriol (E3).

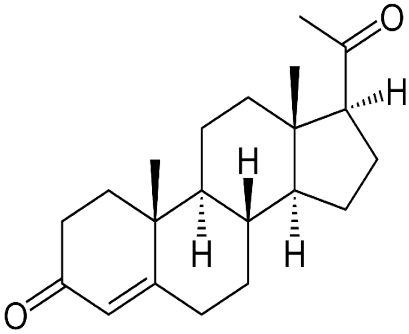

Figure 4: Progesterone (P4).

Experimental

Materials and chemicals

The reference standards of Estrone, Estradiol, and Estriol were acquired from Humco Holding Group (Texarkana, TX); and Progesterone reference standard was acquired from Spectrum Chemicals (New Brunswick, NJ). Potassium phosphate monobasic monohydrate, Acetonitrile, and Methanol were purchased from Fisher Scientific (Fairlawn, NJ). The analytical column was purchased from Phenomenex® (Torrance, CA). HRT base placebo was supplied by Humco Compounding (Texarkana, TX). A Mettler-Toledo pH meter was used for the pH determination of buffer and AWS, AL-201S analytical balance was also used during method development and validation study.

Instrumentations and chromatographic conditions

A Waters 2695 HPLC System with a photodiode array detector (PDA) was used for method development, validation, and potency analysis. The data was acquired and processed using Water's Empower 2 software. A Phenomenex® Gemini C-18 (150 × 4.6 mm C18 5 µm Part # 00F-4435-E0) column was used for this analysis at ambient temperature. A 0.03M Potassium Phosphate monobasic monohydrate buffer with adjusted pH 6.4 and acetonitrile was pumped through the column at a flow rate of 1.0 mL/min. The gradient program was applied as per Table 1. A 10 µL injection volume was used for the method. Signal detection was carried out at 225 nm wavelength and chromatographic run time was set up at 23 minutes. Acetonitrile (ACN): purified water (70:30 v/v) was used as diluent. Approximate retention time of each analytes is given in the Table 2.

Preparation of reagents/solutions

Mobile phase and diluent preparation: A 4.08 g portion of potassium phosphate monobasic monohydrate salt was weighed, and 1000 mL of purified water was added and mixed with stir bar. The buffer solution's pH was adjusted to 6.4 ± 0.05 with 5N KOH. Finally, the buffer solution was filtered through 0.45 µm diameter polytetrafluoroethylene (PTFE) filter using vacuum filtration system and degassed. This is solution A. Acetonitrile is the solution B for the mobile phase. Moreover, diluent was prepared by combining acetonitrile and water (70:30 v/v).

Standard preparation: Four female hormones (Progesterone P4, Estrone E1, Estradiol E2, and Estriol E3) stock standard solution (0.5 mg/mL) was prepared by weighing respective reference standards (50.0 ± 5 mg) and transferring into a 100 mL volumetric flask. About 50 mL of diluent was added to the flask. The volumetric flask was sonicated until all standards were completely dissolved. The aliquot was diluted to volume with diluent. This is the stock standard solution. A 10.0 mL portion of stock standard solution was pipetted into a 50 mL volumetric flask. The working standard solution was diluted to volume with diluent, and the final working standard concentration was 0.1 mg/mL.

Sample and placebo preparation: All hormone concentrates were separately weighed (target weight ± 10%) into separate 100 mL volumetric flask. Hormone concentrate samples were prepared in such a way that the final concentration of each active was maintained as 0.1 mg/mL. The target weights of Progesterone 40%, Estrone 1%, Estradiol 10%, and Estriol 10% hormone concentrates were: 0.25 g, 1.0 g, 0.5 g, and 0.5 g respectively. Samples were weighed into each of 100 mL volumetric flasks. About 50 mL of diluent was added to each of the flasks and sonicated until the samples were fully dispersed. Aliquots were equilibrated to the room temperature prior to dilute to the volume with diluent and mix. These are the stock sample solutions. The working sample solution for each of the formulation was prepared by pipetting as follows: 5 mL to 50 mL volumetric flask (P4), no further dilution (E1), 10 mL to 50 mL vol. flask (both E2 and E3). The flasks were then diluted to the volume with diluent and mixed well. Approximately 3 mL of each of the sample was filtered using a 0.45 µm PTFE Teflon syringe filter into the appropriate HPLC vial for the sample solution analysis. For placebo preparation, about 0.5 g of the available placebo was weighed into 100 mL volumetric flask. Similar sample preparation procedure was followed as E2/E3 sample.

Time	% A Buffer (v/v)	% B ACN (v/v)	Elution
0.00	75.0	25.0	Isocratic
4.00	75.0	25.0	Isocratic
18.00	10.0	90.0	Gradient
18.01	75.0	25.0	Isocratic

Table 1: Gradient program for the HPLC Analysis.

Hormones	Approximate Retention Time
Estriol (E3)	7.5 – 8.5 min
Estradiol (E2)	12.2 – 13.2 min
Estrone (E1)	13.2 – 14.2 min
Progesterone (P4)	15.8 – 16.8 min

Table 2: Approximate retention time of analytes.

Analytical method development and validation: A novel analytical method was developed for the determination of four female hormones and number of chromatographic conditions were adjusted to make the method 'robust'. Method development includes but not limited to adjustment the buffer pH, mobile phase flow rate, detection wavelength, mobile phase gradient programing, and column selection. System suitability acceptance criteria were determined for elements including: retention time, theoretical plates, tailing factor, resolution were also studied prior to validation of the method. Figures 5-11 are the representative chromatograms of the final developed method.

System Suitability

System suitability criteria was set prior to the method validation study. The criteria include: the duplicate standard (standard check) must have a comparison of 98-102% recovery while comparing against regular standard preparation. The % relative standard deviation of the peak area responses of each of the hormones for five consecutive injections at the beginning of the run for the working standard solution must be ≤ 2%. The overall % relative standard deviation of the peak area responses of each hormone in the working standard solution (including bracket standard) must be ≤ 2%. The theoretical plates for each hormone's peak in the working standard solution must be ≥ 2000. The tailing factor for each hormone's peak in the working standard solution must be ≤ 2.0. The resolution between any two hormone peaks in standard solution must be ≥ 1.5. Similarly, the resolution between any other components' peaks (e.g. a placebo peak or a blank peak, if any) and the hormones' peaks in the sample solution must be ≥ 1.5. There should be no co-elution or interference of any peaks with any of the analytes' peaks.

Linearity

The linearity of an analytical procedure is its ability to elicit test results that are directly proportional to the concentration of the analyte in samples over a specified range. Linearity was conducted over a range of about 50% to 150% of the nominal standard concentration (0.1 mg/mL) of each hormone in the working standard solution. A minimum of five concentrations (0.05 mg/mL, 0.08 mg/mL, 0.10 mg/mL, 0.12 mg/mL, and 0.15 mg/mL) were tested and calibration curves were plotted by taking the peak area curve on the Y-axis and the concentration (mg/mL) on the X-axis. The specification was set up for the linearity as: the coefficient of determination (r^2) from the plotted area response versus concentration curve must be ≥ 0.99. The average percent recovery must be 98-102% of the amount prepared over about 50-150% of the nominal standard concentration in each active.

Accuracy

The accuracy of an analytical procedure is the closeness of test results obtained by that procedure to the true value. The accuracy of this method was verified by determining the recovery of a known amount of each analyte added to the sample matrix (a spiked placebo).

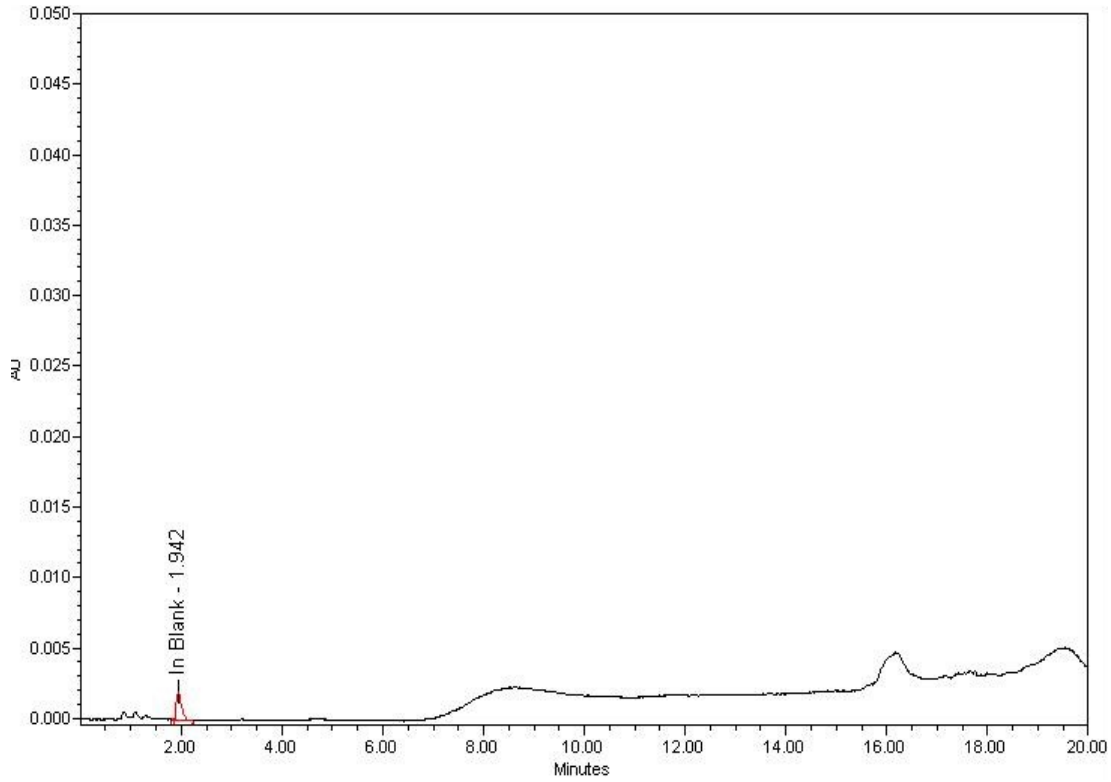

Figure 5: Blank Chromatogram (Diluent).

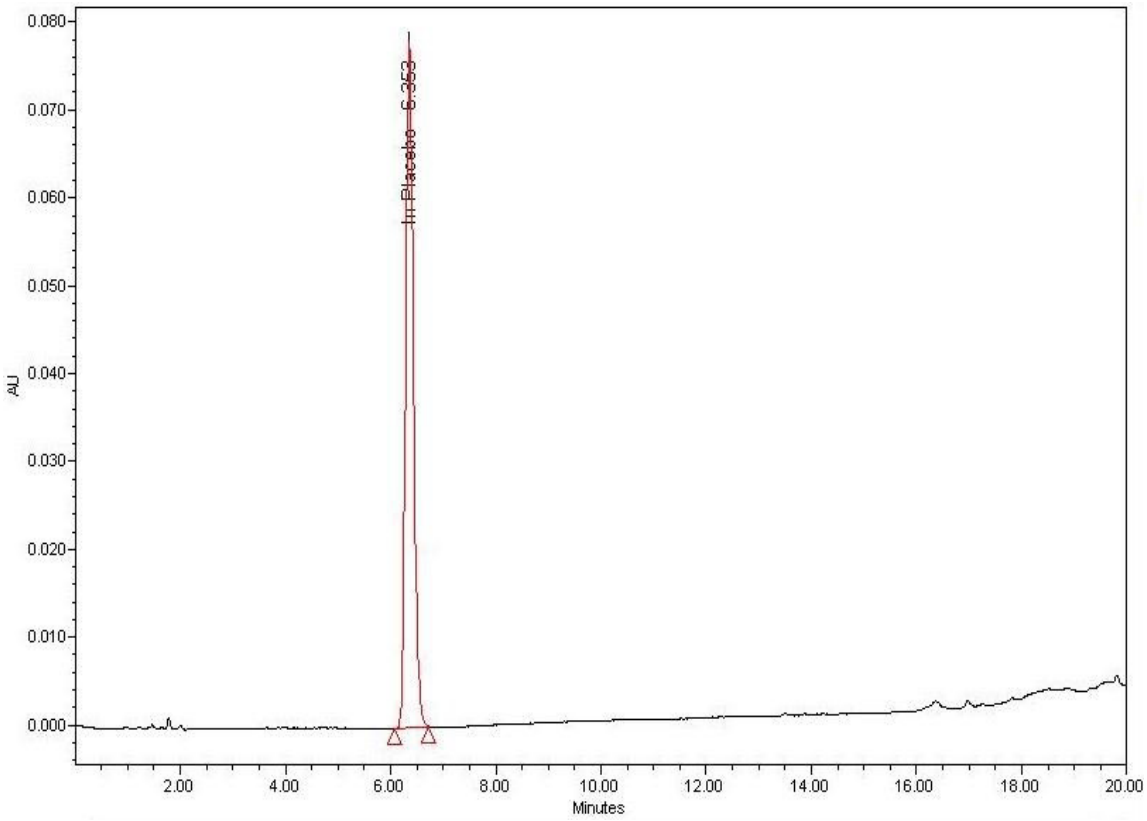

Figure 6: Placebo (HRT Heavy) Chromatogram.

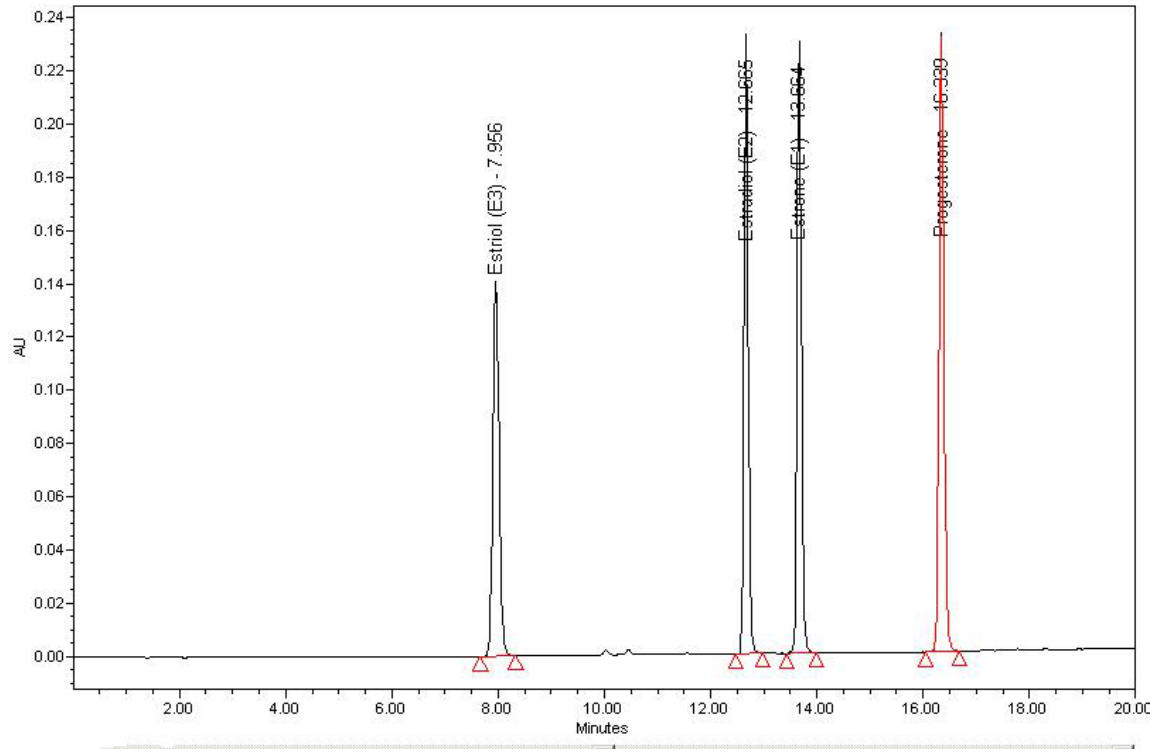

Figure 7: Working Standard Solution Chromatogram of P4, E3, E2, and E1.

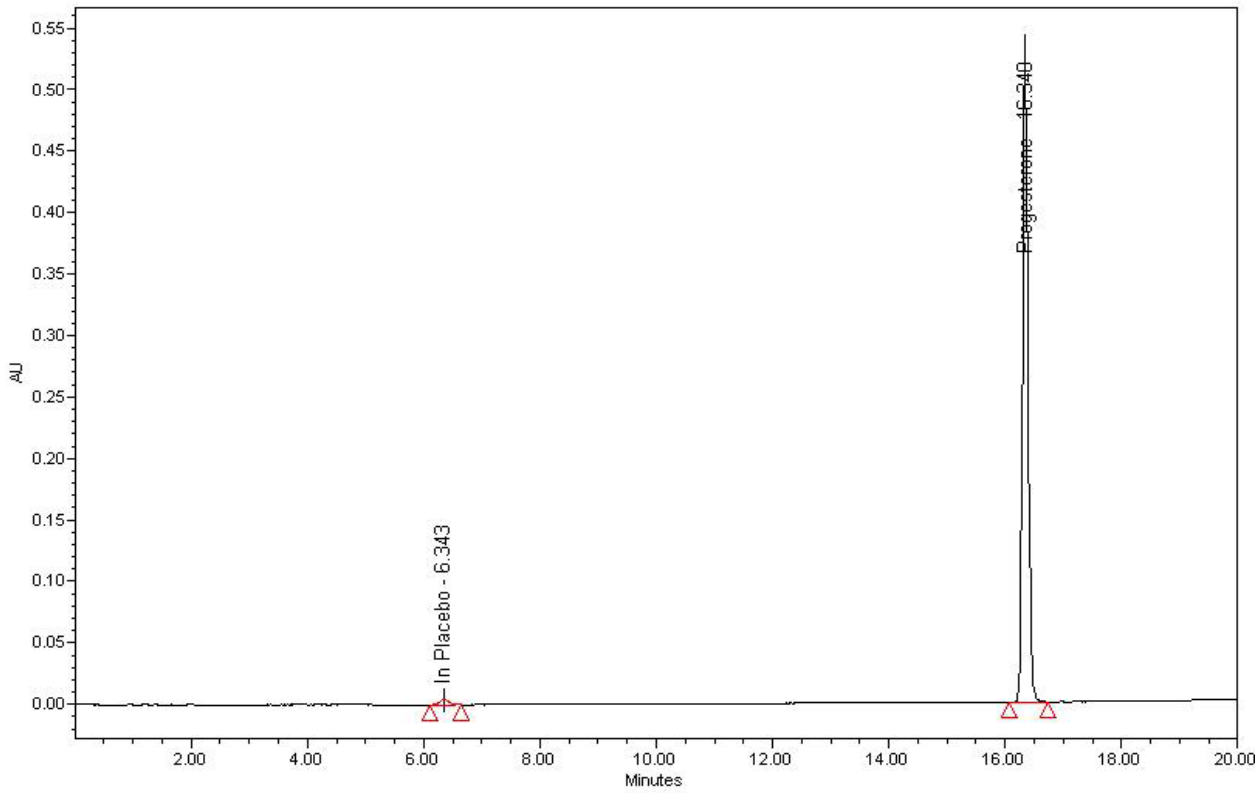

Figure 8: Progesterone (P4) in hormone concentrates.

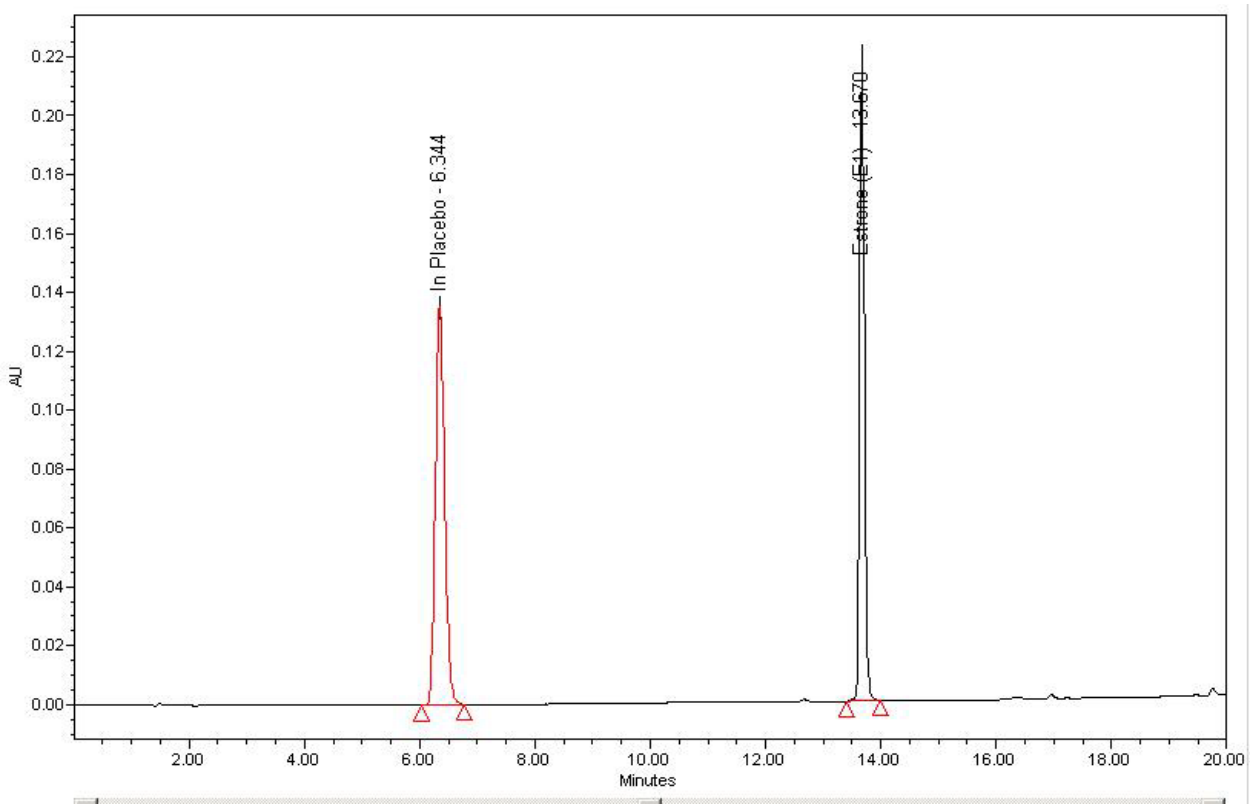

Figure 9: Estrone (E1) in hormone concentrates.

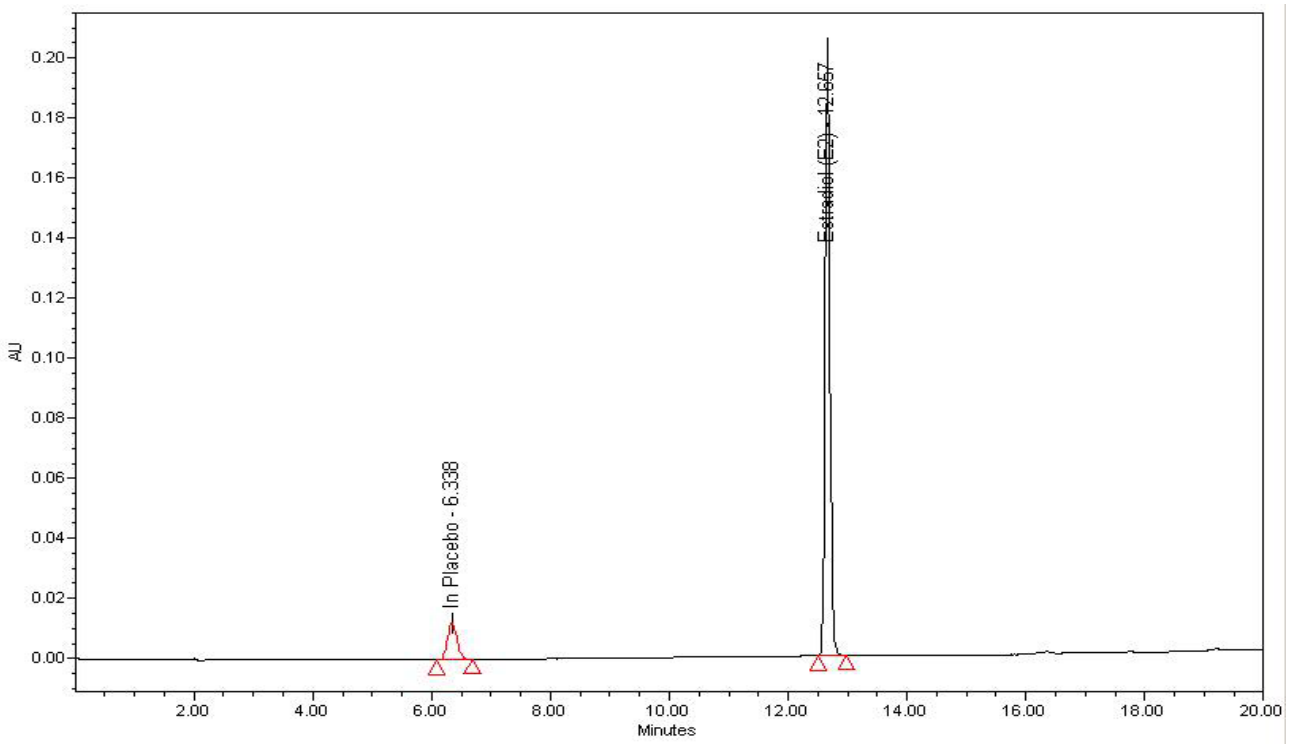

Figure 10: Estradiol (E2) in hormone concentrates.

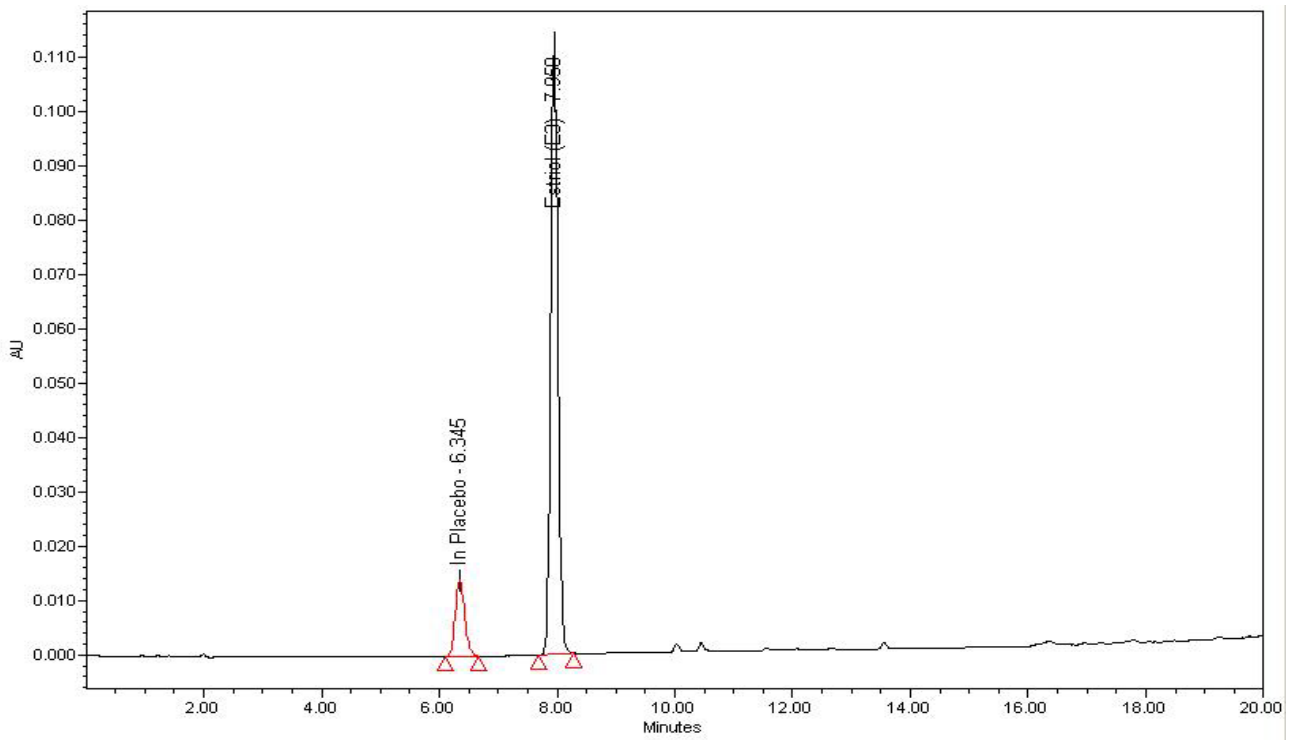

Figure 11: Estriol (E3) in hormone concentrates.

Accuracy solutions were prepared by spiking in the appropriate amount of the analytes of interest into the sample matrix and assaying using a standard. A stock solution of HRT heavy base placebo was prepared and spiked appropriate amount with the known amount of analyte of interest (Progesterone P4, Estrone E1, Estradiol E2, and Estriol E3). Accuracy samples were prepared by serial dilutions of the stock standard added to the placebo solutions at 80%, 100%, and 120% of the nominal analyte concentrations. Three solutions of each active in each accuracy level were prepared by spiking in the appropriate amount of actives (separately) into the placebo. These solutions were prepared in triplicate for a total of 9 solutions each (36 accuracy solutions for 4 hormones) with triplicate injections. The data were evaluated the amount prepared versus the amount recovered and expressed as a percentage recovery.

Specificity

The specificity is the ability to assess unequivocally the analyte of interest in the presence of those components which may be expected to be present in the sample matrix. Specificity/selectivity is the most crucial parameter of any analytical method used for stability and assay determination. The specificity of this method was determined by ensuring the absence of interference by any peaks in the blank diluent or placebo matrix, and resolving any possible degradation peaks from the peaks of interest. For the forced degradation study, the individual pure hormones, hormone concentrate samples, and the HRT Heavy placebo matrix were all subjected to stress conditions (acidic, basic, oxidative, and thermal conditions). The stress conditions for the degradation study comprised of heat (90°C), acid hydrolysis (1N hydrochloric acid), base hydrolysis (1N potassium hydroxide), and oxidation (0.3% hydrogen peroxide), with each condition applied for a period of 24 hours. Acid and base hydrolysis samples were neutralized prior to diluting to the volume with diluent. Oxidation and thermal stress samples were diluted with diluent and all aliquots were filtered prior to analysis by HPLC. Percent assay recovery of each of the analytes and each degradant's % of the parent peak were calculated under each degradation condition.

Precision

The precision of an analytical procedure is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogeneous sample. This was conducted with the individually compounded concentrate of the four hormones (Progesterone P4, Estrone E1, Estradiol E2, and Estriol E3) concentrates. Precision has been further broken down into 'analytical repeatability' and 'intermediate precision'. Analytical repeatability includes the ability of the system to show repeatable measurements (system precision) and the method reproducibility to show reproducible sampling measurements (method precision). The system precision was evaluated by preparing single samples of each of the hormone concentrates and injecting the prepared sample six times. Similarly, method precision was evaluated by preparing six samples of each hormone concentrates. Method precision was repeated for each hormone concentrate formulation by a second analyst as a part of the intermediate precision evaluation. The second analyst prepared and assayed six samples of each hormone concentrate on a different day, using a different instrument, and different column lot number. Assay results of both analysts were combined (n=12) and the percent relative standard deviation (%RSD) obtained.

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations to

the procedure listed in the method and provides an indication of its reliability during normal usage. The robustness of this method was determined by assaying each of the hormone concentrate samples in duplicate while deliberately adjusting the mobile phase flow rate, the pH of the buffer used in the mobile phase, the detector wavelength, and the column temperature. The system suitability parameters (% RSD of peak area response at the beginning and throughout the run, resolution, theoretical plates, and tailing factor) were evaluated after every adjustment to ensure system suitability met the criteria. Average assay results obtained with modified conditions were compared to results obtained using the original method, and the % recovery between the two was determined.

Limit of detection and limit of quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) for four hormones were determined separately as per ICH guidelines. LOD and LOQ of all hormones were obtained on the basis of signal-to-noise ratio by comparing the response of known amount of analyte with respect to the baseline noise. Serial dilutions of analyte solutions were performed and established the minimum concentration at which the analyte can be reliably detected (signal-to-noise ratio of 3:1) and quantitated (signal-to-noise ratio of 10:1).

Results and Discussions

System suitability

All parameters for system suitability were analyzed in each HPLC run for the method validation. The %RSD, average theoretical plates, average tailing factor, average resolution of standard solution, and duplicate standard % recovery (standard check) all met the specific criteria set forth (Table 3).

Linearity

The coefficient of determination, % recovery, and %RSD were obtained from the 50%, 80%, 100%, 120%, and 150% of nominal standard hormones peak area vs. concentration curves. The coefficient of determination r-squared value for each of the hormones was > 0.99. Similarly, % recovery and % RSD were within 98-102% and ≤ 2% meeting the linearity criteria for method validation of all four hormones (Table 4).

Accuracy

Three accuracy levels (80%, 100%, and 120%) of all four hormones were tested as a part of the method validation. Accuracy samples were prepared by spiking known concentration of hormone in the accurate amount of placebo. The average % recovery and average % RSD were determined for each accuracy preparation. The preset criteria for Accuracy for this method (% recovery 98-102% and %RSD ≤ 2%) were met (Table 5).

Precision

Analytical repeatability: Six injections of each separate hormone concentrate formulations were quantified and the %RSD of the assay value was calculated. The % RSD of six injections of each hormone were: Progesterone, 1%; Estrone, 0.2%; Estradiol: 1%, and Estriol: 0.4%. The % RSD of the analyte's assay values for the six injections of the single preparation was less than 1%, meeting the acceptance criteria for the system precision (Table 6).

Six sample preparations of each hormone concentrate were analyzed, and their assay values determined. The %RSD of Progesterone, Estrone, Estradiol, and Estriol were: 1.2%, 1.7%, 1.8%, and 1.2% respectively. The %RSD's of each analytes' assay value for six sample preparations were less than 2%, meeting the specification criteria set forth for the method precision (Table 7).

Intermediate precision: A second analyst repeated the method precision with the same hormone concentrate samples that were quantified by the first analyst. Intermediate precision was performed on a different day, using a different instrument, and a different column lot number. Assay of all four hormones were analyzed and met the % RSD criteria (≤ 2%) for each hormone (Table 8). Each hormone's assay acquired by both analysts were compared, and the combined (n=12) % RSD obtained. The acceptance criteria for the intermediate precision (% RSD ≤ 3%) were met (Table 9).

Robustness

Two samples of each hormone concentrate were prepared and analyzed under both the varied conditions and the normal condition. Average % recovery of the assay values was obtained by comparing the assay results from varied conditions versus the normal condition. Deliberately changed parameters were column temperature (30°C), mobile phase flow rate (0.9 mL/min and 1.1 mL/min), detector wavelength (220 nm and 230 nm), and change in buffer pH (pH 6.35 and 6.45). As shown in the Table 10, all the % recovery assay results under the varied conditions were within 98-102% of the original method condition. The deliberate changes in the method and operational conditions did not affect the chromatograms or the validity of the results; and hence the method is considered robust over that range of conditions.

Specificity

Analysis of blank (diluent) and placebo showed that there are no peaks that interfere (any closer peak must have the resolution of ≥ 1.5) with any of the four female hormones. A blank peak appeared in the solvent front area and additional peak showed up around 20.1 minutes due to change in gradient elution. Similarly, a placebo peak (peak of preservative from the product) appeared at the retention time of 6.2 minutes. None of those peaks interfered with the hormone peaks.

System Suitability Parameters						
Active	**Peak Area %RSD (n=5)**	**Peak Area %RSD (overall)**	**Average Theoretical Plates[a]**	**Average Tailing Factor[a]**	**Average Resolution[a]**	**Standard Check**
Progesterone (P4)	0.9	1.1	209444	0.9	19.3	100.9%
Estrone (E1)	0.3	0.8	201203	1.0	8.4	100.7%
Estradiol (E2)	0.3	0.7	181453	0.9	29.5	101.4%
Estriol (E3)	0.3	0.8	27472	1.0	n/a	100.2%
Acceptance Criteria	NMT[b] 2%	NMT 2%	NLT[c] 2000	NMT 2.0	NLT 1.5	98-102%
Meets?	Yes	Yes	Yes	Yes	Yes	Yes

[a]Expressed as mean from five replicate injections and all bracket injections of system suitability of the standard solution. [b]NMT: not more than. [c]NLT: not less than.

Table 3: System suitability parameters and results of standard injections.

	Progesterone (P4)		Estrone (E1)		Estradiol (E2)		Estriol (E3)	
Linearity % (n=3 inj.)	**Avg. % recovery**	**% RSD**	**Avg. % recovery**	**% RSD**	**Avg. % recovery**	**% RSD**	**Avg. % recovery**	**% RSD**
50	98.8%	1.0%	100.0%	0.8%	99.8%	0.7%	100.2%	0.6%
80	99.8%	1.2%	99.6%	1.1%	99.8%	1.0%	99.6%	1.1%
100	100.4%	0.4%	100.0%	0.1%	100.0%	0.1%	99.9%	0.1%
120	101.5%	1.0%	100.5%	0.2%	100.6%	0.2%	100.5%	0.2%
150	99.1%	0.7%	99.8%	0.5%	99.7%	0.5%	99.8%	0.5%
Determination Coefficients								
r^2=0.998607			r^2=0.999734		r^2=0.999716		r^2=0.999753	

Table 4: Linearity results of four female hormones in five different linearity levels.

Hormones	Accuracy levels	Theoretical conc. (%)	Avg. Actual conc. (%)	Average % recovery	Overall % RSD[a]
Progesterone (P4)	80 %	32.000	32.7850	102.05%	1.2%
	100 %	40.000	40.1990	101.03%	1.0%
	120 %	48.000	47.9138	99.68%	1.2%
Estrone (E1)	80 %	0.8056	0.8121	100.85%	0.3%
	100 %	1.007	1.0013	99.81%	0.4%
	120 %	1.208	1.1867	99.03%	0.8%
Estradiol (E2)	80 %	8.224	8.1419	99.30%	1.1%
	100 %	10.280	10.1456	98.92%	0.8%
	120 %	12.336	12.1321	98.22%	0.9%
Estriol (E3)	80 %	7.928	8.0870	101.38%	0.6%
	100 %	9.910	9.9147	100.42%	0.1%
	120 %	11.892	12.0771	101.59%	0.4%

[a] Triplicate sample preparation and triple injection of each sample.

Table 5: Accuracy results of four hormones quantified in three different accuracy levels.

System Precision (n = 6 injections)			
Hormones	**Avg. Peak Area**	**Avg. Cal. assay (%)**	**Peak Area% RSD**
Progesterone (P4)	798470	41.42	1%
Estrone (E1)	1337310	1.00	0.2%
Estradiol (E2)	1269680	10.04	1%
Estriol (E3)	1205393	9.97	0.4%

Table 6: System precision results of each hormones for this method.

Method Precision Assay (% w/w)				
Method Precision Sample ID	**Progesterone (P4)**	**Estrone (E1)**	**Estradiol (E2)**	**Estriol (E3)**
Method Precision # R1	41.04%	1.01%	9.63%	9.82%
Method Precision # R2	41.44%	1.01%	10.14%	9.96%
Method Precision # R3	41.90%	1.00%	10.01%	10.13%
Method Precision # R4	41.89%	0.98%	10.00%	10.02%
Method Precision # R5	42.54%	1.01%	10.12%	9.87%
Method Precision # R6	41.77%	0.97%	10.00%	9.88%
Avg. Assay % w/w (n=6)	41.76%	1.00%	9.99%	9.95%
% RSD (n=6)	1.2%	1.7%	1.8%	1.2%

Table 7: System precision results of each hormones for this method.

Intermediate Precision (Assay % w/w by Analyst 2)				
Int. Precision Sample ID	**Progesterone (P4)**	**Estrone (E1)**	**Estradiol (E2)**	**Estriol (E3)**
Int. Precision # IP 1	40.47%	1.02%	9.71%	10.13%
Int. Precision # IP2	40.34%	1.04%	9.78%	10.09%
Int. Precision #IP3	40.34%	1.02%	9.79%	10.27%
Int. Precision #IP4	40.48%	1.01%	9.74%	10.17%
Int. Precision # IP5	40.30%	0.99%	9.77%	9.96%
Int. Precision # IP6	40.26%	0.99%	9.74%	10.06%
Avg. Assay % w/w (n = 6)	40.36%	1.01%	9.76%	10.11%
% RSD (n = 6)	0.2%	2.0%	0.3%	1.0%

Table 8: Intermediate precision results acquired by second analyst.

Hormone	Combined Assay (% w/w) (n = 12)	% RSD (n = 12)	Specification	Pass?
Progesterone (P4)	41.06%	2.0%	% RSD (n = 12) NMT 3%	Yes
Estrone (E1)	1.00%	1.9%		Yes
Estradiol (E2)	9.87%	1.8%		Yes
Estriol (E3)	10.03%	1.4%		Yes

Table 9. Combined assay results from analyst 1 and analyst 2.

	Column Temp.	Flow Rate		Wavelength		Buffer pH	
Hormone	30°C	0.9 mL/min	1.1 mL/min	220 nm	230 nm	pH 6.35	pH 6.45
Progesterone (P4)	97.8%	100.8%	99.4%	102.0%	99.2%	97.5%	99.5%
Estrone (E1)	99.6%	98.7%	99.8%	100.2%	99.9%	99.8%	99.5%
Estradiol (E2)	100.3%	99.7%	99.6%	100.8%	99.8%	100.0%	99.7%
Estriol (E3)	100.4%	101.2%	98.6%	100.9%	99.9%	101.4%	99.2%

Table 10: Robustness results acquired by changing parameters with varied conditions.

		Degradation Results of Hormones Only			Degradation Results of the Sample Formulation		
Hormone	**Stress Condition**	**Active Conc. (%)**	**Deg. #1 (%)**	**Deg. # 2 (%)**	**Active Assay (%)**	**Deg. #1 (%)**	**Deg. # 2 (%)**
Progesterone (P4)	Acidic[1]	12.66 %	-	-	34.14 %	0.31%	0.52%
	Basic[2]	7.14 %	2.89%	0.29%	37.57 %	0.44%	0.63%
	Oxidation[3]	40.35 %	-	-	40.35 %	0.31%	-
	Thermal[4]	37.74 %	0.35%	1.71%	40.43 %	-	-
Estrone (E1)	Acidic[1]	0.29 %	-	-	1.24 %	0.55%	1.11%
	Basic[2]	0.25 %	0.41%		1.25 %	0.74%	0.34%
	Oxidation[3]	1.01 %	-		1.02 %	0.58%	0.44%
	Thermal[4]	0.99 %	-		1.02 %	0.45%	1.14%
Estradiol (E2)	Acidic[1]	4.27 %	-	-	9.97 %	-	-
	Basic[2]	3.43 %	0.39%		9.80 %	0.34%	
	Oxidation[3]	9.93 %	-		9.91 %	-	
	Thermal[4]	9.85 %	-		9.93 %	0.34%	
Estrone (E3)	Acidic[1]	6.83 %	-	-	9.96 %	0.30%	-
	Basic[2]	6.28 %	-		9.95 %	-	
	Oxidation[3]	10.07 %	-		10.11 %	0.39%	
	Thermal[4]	9.88 %	0.31%		10.14 %	0.61%	

[1]**Acidic:** 1N HCl; 24 h [2]**Basic:** 1N KOH; 24 h [3]**Oxidation:** 0.3% H_2O_2; 24 h
[4]**Thermal:** 90°C; 24 h No degradation detected is denoted by ' – '.

Table 11: Forced deg. results of hormones assay and degradants of actives and concentrates.

Forced degradation results of all the hormones, placebo, and concentrate samples were acquired. Assay results and all the degradants of each active and formulation were tabulated in the Table 11. In most of the stress conditions, all the hormones appeared to be more stable in the concentrate formulations as compared to the hormones by itself. This stability indicating method showed that none of the degradants listed in the table interfered with any of the four hormone analytes. No major degradations of placebo samples were noted in any of the four stress conditions.

Range

The range for this method was established by examining precision, accuracy, and linearity. The method showed that four female hormones are linear at 50-150% and accurate over 80-120% of the nominal standard concentration (0.1 mg/mL).

Limit of detection (LOD) and limit of quantitation (LOQ)

The limit of detection (LOD) of four hormones for this method was determined as: Progesterone: 0.6 µg/mL, Estrone: 0.6 µg/mL, Estradiol: 0.6 µg/mL, and Estriol: 0.9 µg/mL. Conclusion was made by analyzing the peak responses vs the baseline noise (signal- to- noise) ratio about 10:1. Similarly, the limit of quantitation (LOQ) of all four hormones for this method was also determined as: Progesterone: 1.2 µg/mL, Estrone: 1.2 µg/mL, Estradiol: 1.2 µg/mL, and Estriol: 1.7 µg/mL. LOQ was determined by analyzing signal- to- noise ratio about 3:1.

Standard solution stability

Aged but refrigerated (2-8°C) hormone stock standard solution of four female hormones was pipetted and quantified against freshly prepared hormone standard solution containing all four hormones. Standard solution of four female hormones (P4, E1, E2, and E3) in this method has been proven to be stable for at least 7 days when refrigerated below 10°C.

Conclusion

A novel, accurate, simple, and robust RP-HPLC – PDA method for the determination of four female hormones: Estrone, Estradiol, Estriol, and Progesterone in hormone concentrate topical cream has been developed and validated. The method was validated with respect to specificity, sensitivity, linearity, accuracy, precision, robustness, sample

and standard solution stability as per ICH Q2 (R1) guidelines. All the acceptance criteria set forth were met for the analysis of hormones in Humco's hormone concentrate formulations. The blank, excipients, and possible degradation peaks from the formulation were found to be non-interfering with the primary four hormones peaks. This validated method can be applied for the routine analysis of the four hormones as an APIs, individual hormone concentrates, and combined formulation.

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