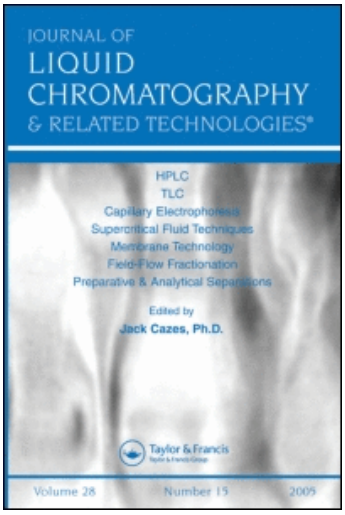

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Determination of a Novel Assay for Pregn-4-ene-3,20-dione in a Gel Formulation Using High-Performance Liquid Chromatography

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Determination of a Novel Assay for Pregn-4-ene-3,20-dione in a Gel Formulation Using High-Performance Liquid Chromatography

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Abstract: A reversed-phase high performance liquid chromatographic (RP-HPLC) method was developed and validated for the determination of pregn-4-ene-3,20-dione in a gel formulation. The chromatographic separation was achieved with methanol-water (75:25, v/v) as mobile phase, a C_{18} column (250 × 4.6 mm; 10 μm), and UV detection at 254 nm. The calibration curve was linear ($r^2 = 0.9999$) from 25–150% of the analytical concentration of 40 μg/mL. The mean percent relative standard deviation values for intra- and interday precision studies were less than 1%. The recovery ranges were 98.61–100.76% from a gel formulation. The limits of detection and quantitation were determined to be 10 and 20 ηg/mL, respectively. The method was specific and successfully routinely used in quality control for analysis of pregn-4-ene-3,20-dione bulk gel samples and final product release.

Keywords: Reversed-phase chromatography, Pregn-4-ene-3,20-dione, Method development, Method validation

INTRODUCTION

Pregn-4-ene-3,20-dione (Figure 1) is a steroidal hormone that plays a central role in the female reproductive cycle. The sex hormone is produced primarily by the adrenal glands, the placenta, and the corpus luteum of the ovaries. In the absence of oocyte fertilization during ovulation, natural pregn-4-ene-3,20-dione levels in the blood serum decline, and menstruation begins. However, if the egg is fertilized, pregn-4-ene-3,20-dione levels will increase to

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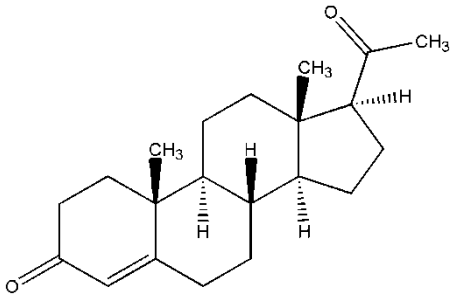

Figure 1. Chemical structure of pregn-4-ene-3,20-dione.

support the pregnancy, maintain the corpus luteum, and promote mammary gland development and milk production. Whether synthesized or extracted, pregn-4-ene-3,20-dione features 21 carbons, 30 hydrogen, and 2 oxygen atoms per molecule and a molecular weight of 314.47.

Pregn-4-ene-3,20-dione consists of four interconnected cyclic hydrocarbons and contains ketone and oxygenated functional groups, as well as two methyl branches. Like all steroids, it is hydrophobic. This is mostly due to its lack of very polar functional groups. Pregn-4-ene-3,20-dione is synthesised from pregnenolone, a derivative of cholesterol. This conversion takes place in two steps. The 3-hydroxyl group is converted to a keto group and the double bond is moved to C-4 from C-5.

Pregn-4-ene-3,20-dione ($C_{21}H_{30}O_2$), forms a white, odourless, crystalline powder or colourless crystals, that are stable in air, practically insoluble in water, and soluble in organic solvents such as alcohol, acetone, and dioxane. The melting point for crystalline pregn-4-ene-3,20-dione is between 126°C and 131°C. Naturally occurring pregn-4-ene-3,20-dione aids glucose metabolism, and facilitates the formation of healthy bones. In addition, synthetic derivatives of this hormone is prescribed for birth control purposes, as well as to alleviate various difficulties associated with menopause. With hormone replacement therapy, pregn-4-ene-3,20-dione helps reduce the risk of endometrial cancer inherent in estrogens therapy. However, blood clots, various cardiovascular disorders, and decreased high density lipoprotein levels have also been conditions associated with progestin administration.

Pregn-4-ene-3,20-dione is also widely used in therapy treatment. The most frequent therapeutic uses of pregn-4-ene-3,20-dione are for dysfunctional uterine bleeding or amenorrhoea,[1,2] for contraception (either alone or with, e.g., estradiol or mestranol in oral contraceptives), and in combination with estrogens for hormone replacement therapy of post menopausal women.[3,4]

Pregn-4-ene-3,20-dione occupies a key position in the synthetic pathway, which leads to the formation of cortisol and cortisone in the adrenal gland. In addition, it is an intermediate in the biosynthesis of androstenedione in the adrenal gland[5] and in the ovary.[6] Chromatography has already been

widely applied to the steroids, both for preparative and analytical work.[7] However, an HPLC assay method has so far not been reported for pregn-4-ene-3,20-dione. The purpose of this research was to develop and validate a robust, accurate, sensitive, and simple HPLC method for the quantitation of pregn-4-ene-3,20-dione in a gel formulation for bulk and final product release. Thereafter, this method was successfully applied for the separation and quantification study of this important compound in the gel formulation.

EXPERIMENTAL

Materials

All chemicals and reagents were of the highest purity. HPLC grade methanol was obtained from Merck (Darmstadt, Germany). Pregn-4-ene-3,20-dione reference standard was purchased from Sigma chemicals (St. Louis, MO, USA). Deionised distilled water was used throughout the experiment.

HPLC Instrumentation and Conditions

A PerkinElmer (Norwalk, CT) HPLC system equipped with a module series 200 UV-vis detector, series 200 LC pump, series 200 autosampler, and series 200 peltier LC column oven were used in this research study. The data were acquired via PE TotalChrom Workstation data acquisition software, (v. 6.2.0) using PE Nelson series 600 LINK interfaces. All chromatographic experiments were performed in the isocratic mode. The second instrument used in intermediate precision study was also a PerkinElmer HPLC system. The mobile phase consisted of a mixture of methanol-water (75:25, v/v). The flow rate was set to 1.5 mL/min and the oven temperature to 40°C. The injection volume was 20 μL and the detection wavelength was set at 254 nm. The chromatographic separation was carried out on a 250 × 4.6 mm, 10 μm C_{18} μ-Bondapak column obtained from Waters (Milford, MA).

Standard Preparation

An accurately weighed amount (200 mg) of pregn-4-ene-3,20-dione reference standard was placed in a 50 mL volumetric flask and dissolved in methanol (stock). A 10 mL aliquot of stock solution was diluted to 100 mL in methanol. Further, a 10 mL aliquot solution was diluted to 100 mL in methanol, yielding a final concentration of 40 μg/mL.

Sample Preparation

An accurately weighed amount (40 mg) of pregn-4-ene-3,20-dione sample gel was dissolved in 100 mL methanol and sonicated for 10 minutes (stock). A 10 mL aliquot of stock gel sample solution was diluted to 100 mL in methanol, yielding a final concentration of 40 μg/mL.

RESULTS AND DISCUSSION

Method Development

The chromatographic analysis of pregn-4-ene-3,20-dione was carried out in the isocratic mode using a mixture of 75% methanol in water (75:25, v/v) as mobile phase. The column was equilibrated with the mobile phase flowing at 1.5 mL/min for 30 min prior to injection. The column temperature was held at 40°C. Standard and sample solutions of 20 μL were injected automatically into the column. The optimal wavelength for pregn-4-ene-3,20-dionedetection was established using two UV absorbance scans over the range of 190 to 400 nm, one scan of the mobile phase, and the second of the analyte in the mobile phase. It was shown that 254 nm is the optimal wavelength to maximise the signal. Chromatograms of the pregn-4-ene-3,20-dione gave good peak shape (Figure 3a & 3b) and co-elution of excipients was not observed (Figure 3c) at the same retention time as pregn-4-ene-3,20-dione. The retention time for pregn-4-ene-3,20-dione was 4.20 minute. To evaluate the quantitative nature of the analytical method, a series of samples with different amounts of pregn-4-ene-3,20-dione were run to investigate the best assay concentration. Using a C_{18} column, best concentration was assessed by injecting five reference standard of pregn-4-ene-3,20-dione in the range of 0.001–0.20 mg/mL. The integrated peak areas were plotted versus amount injected. The calibration curve was found to be linear from concentration range 0.025–0.15 mg/mL with a correlation coefficient of 0.9999. On the bases of these data, the best concentration (40 μg/mL) was chosen as a working concentration for the assay.

System Suitability Test

The accuracy and precision of HPLC data collected begin with a well behaved chromatographic system. The system suitability specifications and tests are parameters that provide assistance in achieving this purpose. In the present research, parameters that were used in HPLC assay method development are discussed.

Capacity Factor (k′)

Capacity factor (also called retention factor) is a measure of the retention time of a compound in the sample with a given combination of mobile phase and column. It is defined as $k'_{(A)} = (t_A - t_0)/t_0$ in which t_A is the retention time of the compound and t_0 refers to retention time for an unretained compound. In the present study, t_0 was 4.20 min. For an optimum separation, the retention factor should be in the range of $0.5 < k' < 10$. Calculated k' value was 4.32 for the major component of pregn-4-ene-3,20-dione.

Selectivity Factor (α)

Selectivity parameter is a measure of separation of two compounds A and B; it is defined as $\alpha = k'_A/k'_B$ (k' is the respective capacity factor). Therefore, it is the ratio of the relative retentions of two compounds. In the present study, the calculated selectivity parameter for separation of pregn-4-ene-3,20-dione and minor impurity was 1.6.

Tailing Factor (T)

Tailing factor (T) refers to peak asymmetry. Many chromatographic peaks do not appear in the shape of normal Gaussian distribution. Therefore, tailing factor should be calculated using the following equation for chromatographic peaks: $T = w_{0.05}/2f$ in which $w_{0.05}$ is the distance from the leading edge to the tailing edge of the peak measured at a point 5% of the peak height from the baseline and f is the time from width start point at 5% of peak height to retention time. A tailing factor of 1 refers to a symmetric peak. The calculated value of 1.02 was obtained for the major component of pregn-4-ene-3,20-dione peak, which is in the acceptable range of $0.5 \leq T \leq 2$.

Resolution (R_s)

Resolution (R_s) is a measure of how well two peaks are separated. For reliable quantitation, well separated peaks are essential for quantitation. This is a very useful parameter if potential interference peak (s) may be of concern. The closest potential eluting peak to the analyte should be selected. R_s is minimally influenced by the ratio of the two compounds being measured. R_s of >2 between the peak of interest and the closest potential interfering peak (impurity, excipient, degradation product, internal standard, etc.) are desirable. The following equation is used to calculate the resolution: $R_s = (t_{R2} - t_{R1})/(1/2)$

$(t_{w1} + t_{w2})$, where t_w is peak width measured at baseline of the extrapolated straight sides to baseline. In the present work, the resolution value for separation of major component of pregn-4-ene-3,20-dione and minor impurity was 2.62.

Theoretical Plate Number (N)

Theoretical plate number (column efficiency) was also investigated. In a particular separation, column efficiency refers to the performance of the stationary phase. It means how well the column is packed. There are several methods to measure the column efficiency that may or may not be affected by chromatographic anomalies, such as tailing or fronting. In the present study, the number of theoretical plates was calculated using the following equation: $N = 16\ (t_R/t_w)^2 = L/H$. The theoretical plate number depends on elution time but in general should be >2000. N is fairly constant for each peak on a chromatogram with a fixed set of operating conditions. H, or the height equivalent of a theoretical plate (HETP), measures the column efficiency per unit length (L) of the column. Parameters, which can affect N or H, include peak position, particle size in column, flow rate of mobile phase, column temperature, viscosity of mobile phase, and molecular weight of the analyte. For the column used in this study the N value of 5418 is obtained for pregn-4-ene-3,20-dione.

Precision/Injection Repeatability

Injection precision expressed as relative standard deviation (RSD) indicates the performance of the HPLC that includes the plumbing, column, and environmental conditions, at the time the samples are analysed. It should be noted that sample preparation and manufacturing variations are not considered. RSD of $\leq 1\%$ for $n \geq 5$ is desirable. In the present study, injection repeatability was performed by injecting six replicate injections of a solution containing 40 μg pregn-4-ene-3,20-dione/mL. The %RSD of the retention time (min) and peak area were found to be less than 0.29%.

General Recommendations

System suitability testing is essential for the assurance of the quality performance of the chromatographic system. The amount of testing required will depend on the purpose of the analytical method. For dissolution or release profile test methods using an external standard method, K′, T, and RSD are minimum recommended system suitability tests. For acceptance, release,

stability, or impurities/degradation methods using external or internal standards, K′, T, Rs, and RSD are recommended as minimum system suitability testing parameters. In practice, each method submitted for validation should include an appropriate number of system suitability tests defining the necessary characteristics of that system.

Robustness

Robustness studies were also performed that showed 20 μL injection volume was reproducible and the peak response was significant at the analytical concentration (40 μg/mL) chosen. During the robustness study, a number of chromatographic parameters were investigated such as flow rate, temperature, mobile phase composition, and columns from different lots. In all cases, good separations of pregn-4-ene-3,20-dione were always achieved, indicating that the analytical method remained selective for the pregn-4-ene-3,20-dione component under the tested conditions.

Method Validation

Validating analytical methods is a critical component of successful product development, testing, and quality. All raw materials, bulk, and formulated products used in the pharmaceutical formulations require some level of evaluation and testing. Critical decisions may be made based on these results, making it imperative that pharmaceutical and biotechnology companies ensure their accuracy and reproducibility to remain compliant with regulatory guidelines.[8–14] As a best practice, always follow the systematic approach[15] for method validation, the step by step written and approved protocol.[16,17]

Linearity

Linearity was studied in the concentration range 0.025–0.15 mg/mL (25–150% of the theoretical concentration in the test preparation, $n = 3$), and the following regression equation was found by plotting the peak area (y) versus the pregn-4-ene-3,20-dione concentration (x) expressed in mg/mL: $y = 451.95x - 146.62$ ($r^2 = 0.9999$) (Table 1). The demonstration coefficient (r^2) obtained for the regression line demonstrates the excellent relationship between peak area and concentration of pregn-4-ene-3,20-dione (Figure 2). The analyte response is linear over the range of 80 to 120% of the target concentration for pregn-4-ene-3,20-dione.

Table 1. Linearity assessment of the HPLC method for the assay of pregn-4-ene-3,20-dione

Concentration (mg/mL)	Percent of nominal value	Average peak area (μVs) ($n = 3$)	RSD (%)
0.025	25	11083	0.03
0.05	50	22458	0.01
0.075	75	33643	0.01
0.10	100	45367	0.07
0.15	150	67496	0.04

Correlation coefficient: $r^2 = 0.9999$
Equation for regression line: y = 451.95x − 146.62

Accuracy/Recovery Studies

The accuracy of the method was evaluated by means of recovery assay, adding known amounts of pregn-4-ene-3,20-dione reference standard to a known amount of gel formulation, in order to obtain three different levels (75%, 100%, and 125%) of addition. The samples were analysed and the mean recovery was calculated. The data presented in Table 2 shows the recovery of pregn-4-ene-3,20-dione in spiked samples met the evaluation criteria for accuracy (100 +/−2.0% over the range of 80 to 120% of target concentrations).

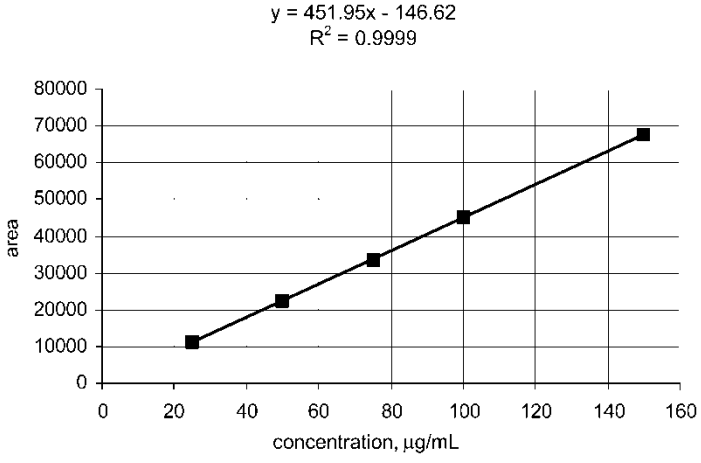

Figure 2. Calibration of pregn-4-ene-3,20-dione concentration with peak area. Range of 25–150 μg/mL.

Table 2. Recovery studies of pregn-4-ene-3,20-dione from samples with known concentration

Sample	Percent of nominal	Amount of pregn-4-ene-3,20-dione (mg)		Recovery (%)	RSD (%)
		Added	Recovered		
1	75	3.170	3.194	100.76	0.73
2	75	3.179	3.156	99.28	
3	100	3.902	3.882	99.49	0.04
4	100	4.258	4.240	99.58	
5	125	5.039	4.969	98.61	0.20
6	125	5.253	5.195	99.00	
Mean				99.44	

Specificity/Selectivity

Injections of the extracted placebo were performed to demonstrate the absence of interference with the elution of the pregn-4-ene-3,20-dione. These results demonstrate (Figure 3c) that there was no interference from the other materials in the gel formulation and, therefore, confirm the specificity of this method. However, pregn-4-ene-3,20-dione showed degradation after the degradation treatment.

Forced degradation studies were performed to evaluate the specificity of pregn-4-ene-3,20-dione under five stress conditions (heat, UV light, 1M

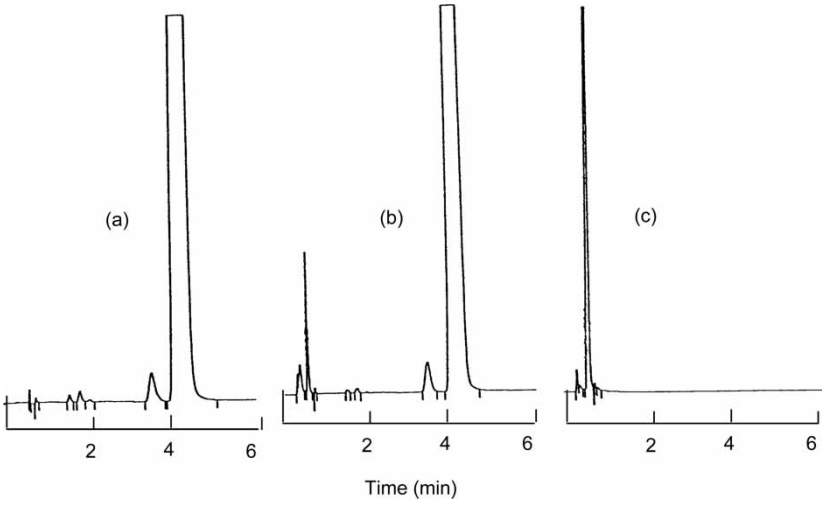

Figure 3. Reversed-phase separation of pregn-4-ene-3,20-dione (a) reference standard, (b) gel sample, (c) placebo formulation. Eluent: 75/25 (v/v) methanol/water at a flow rate of 1.5 mL/min. Column: C_{18}, 250 × 4.6 mm.

Table 3. Summary results of forced degradation study of pregn-4-ene-3,20-dione

Stress conditions	Sample treatment	Retention time (min)	Pregn-4-ene-3,20-dione peak area (μVs) (% of degradation)
Reference	Fresh solution	4.20	No degradation
Acid	1M HCl for 24 hour	4.20	
Base	1M NaOH at 80°C for 1,2,3,5 hour	4.20, 4.20, 4.22, 4.81	8.7, 10.4, 19.4, 81.5
Hydrogen peroxide	3% solution	4.20	No degradation
Heat	50°C for 1 hour	4.21	No degradation
Light	UV Light for 24 hour	4.20	No degradation

hydrochloric acid, 1M sodium hydroxide, hydrogen peroxide). The study showed that degradation of pregn-4-ene-3,20-dione only occurred under alkaline conditions and no degradation was observed in acidic or oxidising conditions. Increased stress conditions were applied by heating pregn-4-ene-3,20-dione solutions in 1M NaOH at 80°C for 1 h, 2 h, 3 h, and 5 h. Degradation was observed, increasing with increased heating time (Table 3).

Precision (Repeatability and Intermediate Precision)

The precision of the method was investigated with respect to repeatability and intermediate precision. Repeatability (intraday precision) of the method was evaluated by assaying six replicate injections of the pregn-4-ene-3,20-dione at 100% of test concentration (40 μg/mL). The %RSD of the retention time (min) and peak area were found to be less than 0.29% (Table 4). The

Table 4. Repeatability study of the HPLC assay of pregn-4-ene-3,20-dione

Sample	Retention time (min)	Peak area (μVs)
1	4.20	15951
2	4.20	15895
3	4.21	15877
4	4.20	15876
5	4.20	15848
6	4.21	15822
Mean	4.20	15878
SD	0.005	43.95
RSD%	0.12	0.28

intermediate precision (interday precision) was demonstrated by two analysts, using two HPLC systems, and evaluating the relative peak area percent data across the two HPLC systems at three concentration levels (50%, 100%, 150%) that cover the assay method range (0.025–0.15 mg/mL). The mean and %RSD across the systems and analysts were calculated from the individual relative percent peak area mean values at the 50%, 100%, and 150% of the test concentration. The %RSD values for both instruments and analysts were $\leq$0.43% (Table 5) and illustrated the good precision of this analytical method.

Limits of Detection and Quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) tests for the procedure were performed on samples containing very low concentration of analyte. LOD is defined as the lowest concentration of analyte in a sample that can be detected above baseline noise. It is expressed as a concentration at a specified signal-to-noise ratio, typically, three times the noise level. LOQ is defined as the lowest concentration of analyte in a sample that can be reproducibly quantitated above the baseline noise that gives a s/n of 10. The LOD was (s/n 3.0) 10 ηg/mL. LOQ was (s/n 10.2) 20 ηg/mL and %RSD for six injections was 0.56%.

Stability of Analytical Solutions

Sample and standard solutions were chromatographed immediately after preparation and then reassayed after storage at room temperature for 24 h. The

Table 5. Intermediate precision data of the HPLC assay of pregn-4-ene-3,20-dione

	HPLC system 1			HPLC system 2		
Sample	S1 (50%)	S2 (100%)	S3 (150%)	S1 (50%)	S2 (100%)	S3 (150%)
Analyst 1	100.54	100.08	99.89	99.62	99.82	99.94
Analyst 2	100.19	100.07	99.73	99.94	100.13	99.97
Mean (HPLC)	100.37	100.08	99.81	99.78	99.98	99.96
Mean (Analyst 1 + 2)	100.07	100.03	99.88			
RSD (criteria $\leq$ 1%) HPLC	HPLC1 S1 + HPLC2 S1 = 0.42; HPLC1 S2 + HPLC2 S2 = 0.07; HPLC1 S3 + HPLC2 S3 = 0.11					
RSD (criteria $\leq$ 1%) Analysts	HPLC1 S1 + HPLC2 S1 = 0.39; HPLC1 S2 + HPLC2 S2 = 0.14; HPLC1 S3 + HPLC2 S3 = 0.11					

Table 6. Stability results of pregn-4-ene-3,20-dione samples and standard solutions ($n = 3$)

Time (h)	Pregn-4-ene-3,20-dione recovery (mg/100 mL)	Percent of initial
Standard		
0	3.955	100.0
24	3.952	99.9
Sample		
0	5.503	100.0
24	5.478	99.5

results given in Table 6 show that there was no significant change (<1% response factor) in pregn-4-ene-3,20-dione concentration over this period.

CONCLUSION

A new and simple HPLC method for the assay of pregn-4-ene-3,20-dione was developed and validated. The results showed that the method is very selective, no significant interference peak was detected; accurate, with the pregn-4-ene-3,20-dione recoveries of 98.61–100.76%, and reproducible with the %RSD less than 1% in all cases. The method was sensitive; as little as 10 ηg/mL could be detected with the LOQ of 20 ηg/mL. The method was used in quality control for analysis of pregn-4-ene-3,20-dione in bulk, raw materials, and final gel product release.

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