DEVELOPMENT AND VALIDATION OF RP-HPLC-DAD METHOD FOR DETERMINATION OF PROGESTERONE IN CAPSULE

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ABSTRACT

Progesterone is a hormone produced in the human body that is essential for regulating many vital functions. A reversed phase-High Performance Liquid Chromatography-Diode Array Detection (RP-HPLC-DAD) method for the quantitative determination of progesterone in pure forms and capsule was developed and validated in the present study. The validation parameters such as linearity, precision, accuracy, specificity, sensitivity and stability were studied according to the International Conference on Harmonization Guidelines. Progesterone was eluated by Agilent C_{18} (5 μm, 150 x 4.6 mm) column and detected at 240 nm. The eluation was achieved isocratically with a mobile phase of acetonitrile-Milli-Q grade water (70:30, v:v) at a flow-rate of 1 mL min^{-1}. The injection volume was 10 μL. The range of quantification was 0.25-5 μg mL^{-1}. Intra-day and inter-day precision expressed as the percent relative standard deviation (RSD %) were less than 2.5 %, and accuracy (relative error) was better than 11.0 % (n = 6). LOQ and LOD values were found to be 0.1 and 0.08 μg mL^{-1}, respectively. The developed method was successfully applied to assay of progesterone in capsule.

Keywords: Progesterone, RP-HPLC-DAD method, capsule

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1. INTRODUCTION

Progesterone also known as P4 (pregn-4-ene-3,20-dione) is a C-21 (Fig.1) **steroid hormone** involved in the **female menstrual cycle**, **pregnancy** (supports ***gestation***) and **embryogenesis** of humans and other species. Progesterone belongs to a class of hormones called **progestogens**, and is the major naturally occurring human progestogen /1/. Progesterone participates in the regulation of the menstrual cycle and is especially important in preparing the uterus for implantation of the blastocyst and in the maintenance of pregnancy /2/.

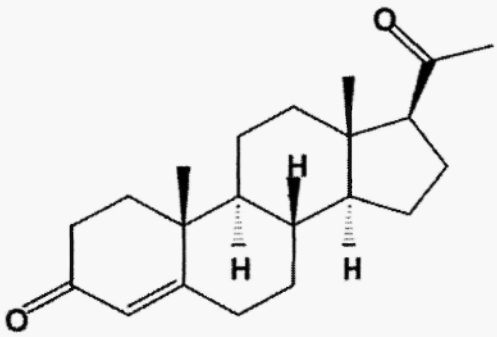

Fig. 1: Chemical structure of progesterone

Progesterone has been administered as part of hormone replacement therapy in menopausal women. Due to first-pass metabolism; side-effects could result in discontinuation of therapy /1,2/. The most frequent therapeutic uses of progesterone are for dysfunctional uterine bleeding or amenorrhoea /3,4/, 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 /5-7/. Progesterone can be administered orally, vaginally, or through intra-muscular injection and all these routes of administration have demonstrated characteristic endometrial histological changes /8/.

The literature shows that gas chromatography (GC), gas chromatography-mass spectrometry (GC-MS) /8-12/ and spectrophotometric methods /13-18/ have been reported for the simultaneous determination of progesterone, 17-hydroxyprogesterone and other 3-keto steroids. Progesterone in pharmaceutical preparations and cosmetics was also determined by means of high-

performance liquid chromatography (HPLC) systems using water-methanol or alcoholic mixtures /13, 18-24/ as mobile phases. The USP method /25/, however, has some disadvantages because the column has to be thermostatted at 40°C, and high percentages of various organic solvents are necessary.

The purpose of this investigation was to work the analysis of progesterone with RP-HPLC-DAD method in pure samples and pharmaceutical preparation. The method was developed by considering all optimization parameters with a simple sample preparation without a complex creation process. The developed method was validated according to International Conference on Harmonization guidelines /26/ for validation of analytical procedures. Then the developed and validated method, which does not involve pretreatment steps, was applied in capsule form.

2. MATERIAL AND METHODS

2.1. Materials and chemicals

Progesterone was purchased from Riedel De Haen. HPLC and analytical grade solvents including ethanol and acetonitrile were purchased from Merck (Germany). Pharmaceutical preparation (Progestan® soft capsule, produced by Kocak Pharm. Ind., Turkey. Progesterone capsules were obtained commercially. The capsules were claimed to contain 100 mg (progesterone/capsule) of drug and TiO_2 as excipient.

2.2. Standard solutions

100 μg mL^{-1} standard stock solution of progesterone was prepared in ethanol. Stock solution was stored at -20°C in a glass flask and brought to room temperature before use. Working solutions (WS) containing 0.25, 0.5, 0.75, 1, 1.5, 2, 3 and 5 μg mL^{-1} of progesterone were daily prepared by diluting the 100 μg mL^{-1} stock solution to a constant volume with ethanol. The working solutions were prepared daily in analysis. The solutions were filtered through a Millipore filter (pore size: 0.45 μm) and transferred to an auto sampler vial for analysis.

Quality control (QC) samples were prepared by adding aliquots of standard solution of progesterone to final concentrations of 0.3, 1.25 and 4 μg mL^{-1}.

2.3. Assay of progesterone in capsules

The contents of five capsules, each containing 100 mg of progesterone, were pulverized using a mortar and pestle. An aliquot of this material, equivalent to 50 mg of progesterone, was accurately weighed and transferred into a 100-mL volumetric flask. Ethanol was added and the flask was shaken for 30 min. The volume was completed to 100 mL, after which the solution was filtered through paper and the first 5-mL discarded. An aliquot of 5 mL of the filtrate was diluted with ethanol to obtain a 5 μg mL^{-1}. Progesterone concentrations of 1 and 2 μg mL^{-1} were prepared from this capsule solution. The solution was then filtered through a 0.22 μm Millipore filter and 10 μL of sample were injected in HPLC employing systems.

2.4. Simulated sample

Simulated samples containing 100 mg of progesterone and 3.3 mg of TiO_2 were prepared and analyzed by the same HPLC procedure. The samples were homogenized (using a mortar and pestle) and the correspondent weight of one capsule was submitted to the analytical procedure.

2.5. Placebo sample

Placebo samples containing 3.3 mg of TiO_2 were prepared and analyzed by HPLC procedure. The samples were homogenized (using a mortar and pestle) and the correspondent weight of one capsule was submitted to the analytical procedure.

3. RESULTS

3.1. RP-HPLC-DAD method

The development of the HPLC method for the determination of drugs has received considerable attention in recent years because of its importance in routine quality control analysis. So, a RP-HPLC-DAD method was proposed as a suitable method for the estimation of progesterone in pharmaceutical dosage form. The chromatographic conditions were adjusted in order to provide a good performance of the assay. The progesterone analysis was performed on a Thermoseparations Spectra HPLC consisted of UV 6000 LP

photodiode array detector (DAD), Series P 400 gradient pump and a Thermoseparations As 3000 autosampler. The method involved C18 column (5 µm, 150 mm x 4.6 mm, Agilent, USA) and a mobile phase consisting of acetonitrile- Milli-Q grade water (70:30, v:v) accomplished at 240 nm. The retention time was 5.24 min at a flow rate of 1 mL min^{-1} and the injection volume was 10 µL. The total run time for an assay was approximately 6 min. Mobile phase selection was based on peak parameters (symmetry and tailing), run time, ease of preparation and cost. The mobile phase was chosen after several trials with other solvent combinations (methanol-water, methanol-acetonitril and acetonitril-water). Figure 2 shows a typical chromatogram obtained from the analysis of a standard and solution of commercial progesterone using the proposed method. As shown in these figures, progesterone was eluted forming symmetrical peak and well separated from the solvent front.

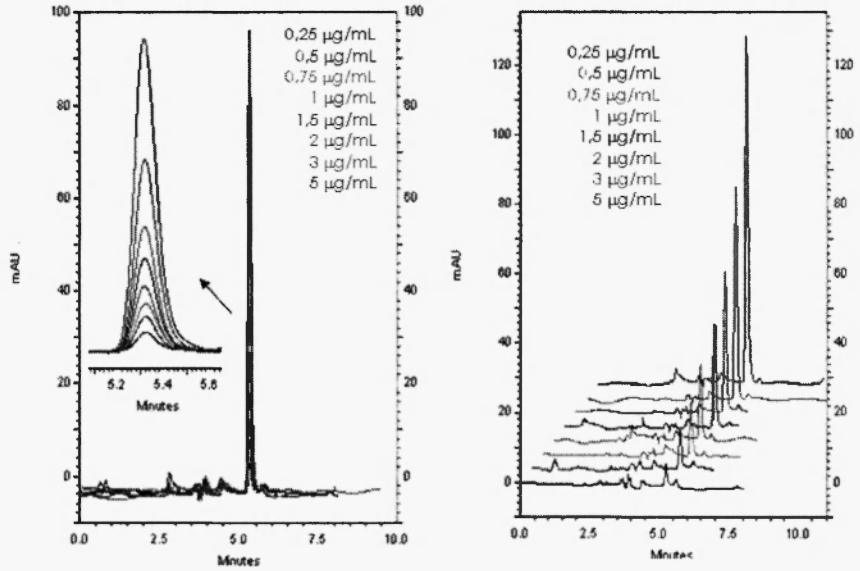

Fig. 2: Chromatograms obtained from calibration graph of a standard solution and a solution of commercial progesterone.

3.2. System suitability

System suitability was performed before conducting the determination for linearity. The system suitability was assessed by six replicate analyses of the

drug at a concentration of 5.0 μg mL^{-1}. The acceptance criterion was ±2% for RSD % for the peak area and retention times for progesterone.

3.3. Linearity/ Range

Linearity of the assay was demonstrated over concentration range of 0.5 to 5 μg mL^{-1} a progesterone in six replicate at eight concentrations (0.25, 0.5, 0.75, 1, 1.5, 2, 3 and 5 μg mL^{-1}). The linearity was evaluated by linear regression analysis, which was calculated by least square regression method. Linear range was determined by plotting the peak area versus sample concentration. The linear regression equation [with standard error of intercept (S_a: 0.0331) and slope (S_b: 0.0491)] and the correlation coefficient, R, was y=1341.0797 x-0.0495 (y: the peak area, x: the concentration of progesterone) and 0.9997, respectively.

To test the sensitivity of the methods in the conditions proposed, the limit of detection (LOD) and limit of quantification (LOQ) were studied. The limit of detection (LOD) which was defined as signal/noise=3 in the method were found to be 0.08 μg mL^{-1}. The limit of quantification (LOQ) which was defined as signal/noise=8 in method were found to be 0.1 μg mL^{-1}. Both accuracy and precision of these values were well within the proposed criteria (RSD % < 20 %).

3.4. Interferences study

In an attempt to detect interference, simulated and placebo samples were prepared and analyzed. Excipient used in this preparation was most commonly used by the pharmaceutical industry. The presence of TiO_2 did not interfere in the results of the analysis (Figs. 3 and 4).

3.5. Precision and Accuracy

The precision of the method as the relative standard deviation (RSD %=100x Standard deviation/Mean) and the accuracy of this method as percent of mean deviation from known concentration [relative error %; (concentration found–known concentration) x 100 / known concentration] were evaluated with intra-day and inter-day measurement at three different concentrations (0.3, 1.25 and 4 μg mL^{-1}) of progesterone.

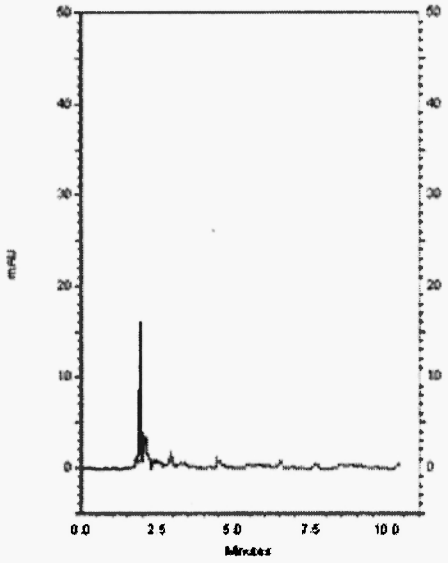

Fig. 3: Chromatogram of placebo sample.

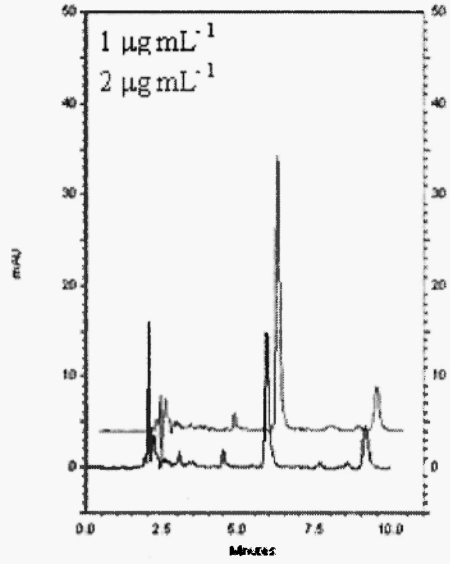

Fig. 4: Chromatograms of commercial sample (Progestan® soft capsule)

Table 1

Precision and accuracy of method (n=6).

λ(nm)	Added (μg mL^{-1})	Intra-day			Inter-day		
		Found±SD (μg mL^{-1})	Precision RSD %[a]	Accuracy	Found±SD (μg mL^{-1})	Precision RSD %[a]	Accuracy
240	0.30	0.33±0.01	1.23	10.14	0.33±0.01	2.02	10.61
	1.25	1.26±0.01	0.82	1.08	1.24±0.02	1.56	-0.52
	4.00	4.13±0.02	0.59	3.27	4.15±0.10	2.37	3.69

SD[a]: Standard deviation of six replicate determinations, RSD: Relative Standard Deviation, Average of six replicate determinations, Accuracy[c] (relative error %) = (added-found)/added x100.

The results of precision and accuracy for progesterone are shown in Table 1. Precision and accuracy of RP-HPLC-DAD method showed acceptable RSD % values and the relative errors %. Intra-day (repeatability) and inter-day (intermediate precision) precision, RSD % values, were lower than1.5 and 2.5 %, respectively. Intra-day and inter day accuracy, relative error %, values were also lower than 11 %. These data indicated that the developed RP-HPLC-DAD method for determination of progesterone had a good precision and accuracy.

3.6. Recovery

The accuracy was determined by recovery of known amounts of progesterone reference standard added to the capsule samples at the beginning of the process. For recovery study, the capsule solutions according to the procedure described at Section *2.3.* were prepared. The standard solutions at 0.3, 1.25 and 4 μg mL^{-1} concentrations of progesterone were transferred in capsule solution to 2 μg mL^{-1} concentration. The final concentrations of these solutions were 2.3, 3.25 and 6 μg mL^{-1}. The peak areas of solutions prepared were determined. The percentage recovery of added progesterone standard was calculated by comparing the found and added concentrations (C_{found}/C_{added}x100) in each case. The mean recoveries and the RSD % values for these recovery values were found in the range of 96.80- 109.6 % and 1.0-1.72 %, respectively. No interference from the common excipient was observed. The results are shown in Table 2.

Table 2
Recovery values of progesterone in capsule (n=6)

Taken (μg mL^{-1})	**Added (μg mL^{-1})**	**Found±SD (μg mL^{-1})**	**Recovery±RSD (%)**
0.30	2.00	2.52±0.10	109.6±1.00
1.25	2.00	3.19±0.15	98.2±1.19
4.00	2.00	5.81±0.28	96.8±1.72

SD: Standard deviation of six replicate determinations, RSD: Relative standard

3.7. Stability

The stability of the standard solution and stock solution was determined by analyzing standard solutions stored in the refrigerator and at room temperature against freshly prepared standards. The percent of standard recovered was used to evaluate stability.

% Standard Y recovered = (area std Y/area fresh std) × (fresh standard concentration/standard Y concentration) × 100

where standard Y = standard being evaluated.

The standard solutions (0.3, 1.25 and 4 μg mL^{-1} concentrations) that were stored for four days under both conditions were found to be stable for three days in the refrigerator and for two days in room temperature, without significant change in concentration (recovery = 100 ± 1%). In addition to this, stock solution was found to be stable for a week in refrigerator. The results are summarized in Table 3.

4. DISCUSSION

The HPLC method is widely employed in pharmacopoeia analysis. For example, the chromatographic procedure was used for the purity characterization and the quantitative determination of drugs. The HPLC analysis of progesterone was reviewed by Shchavlinskii *et al.* /27/ and Maslov *et al.* /28/, but new data have been reported since their studies. An HPLC procedure for the quantitative determination of progesterone in substances and injection solutions was suggested by Tokunaga *et al.* /18/. A liquid chromatography method in commercial pharmaceutical formulations and experimental micellar systems was developed by Pucci *et al.* /20/. This system is based on the C8 (Res Elut, 150x/4.6 mm, I.D. 5 mm,) column, a mobile phase composed of 2-propanol and a pH 2.5, 30 mM phosphate buffer, flow rate 1.5 mL min^{-1} and a UV detection operated at 254 nm. Another HPLC system which was used for the detection of counterfeit drugs by the qualitative and quantitative analysis of nine steroidal drugs (ethynyl-estradiol, diethylstilbestrol, norethisterone, norgestrel, methyltestosterone, medroxyprogesterone acetate, progesterone, testosterone propionate and nilestriol) was based on chromatograph-equipped UV detection, an Alltima C18 column at a flow rate of 1.0 mL $min^{-1}$and using two runs with different mobile phases [methanol–water (62:38, v/v) and (60:40, v/v)] /21/. Maliwal

Table 3
Stability of standard solutions

Added ($\mu g\ mL^{-1}$)	Room temperature				Refrigerated			
	1 Day	2 Days	3 Days	4 Days	1 Day	2 Days	3 Days	4 Days
0.30	106.6	99.8	88.9	75.7	100.4	99.9	98.9	85.9
1.25	100.6	99.9	88.6	76.5	100.7	100.4	97.9	86.5
4.00	100.9	98.2	88.9	76.9	100.2	99.7	97.6	86.3

et al. /13/ determined progesterone in capsule as used in a system based on the C18 (250x/4.0 mm, I.D. 5 μm,) column, a mobile phase composed of methanol and water (80:20, v/v), flow rate 1.0 mL min^{-1}, with manual injection and a UV detection operated at 254 nm. In another work /24/, estriol, estradiol, and estrone, followed by progesterone at pharmacy-compounded drugs containing combinations of the hormones were analyzed on C18 column with isocratic mobile phase of acetonitrile-water (50:50, v/v) at flow rate of 1 mL min^{-1} and column temperature of 30°C. Progesterone was detected at 270 nm.

A simple high-performance liquid chromatography (HPLC) method with ultraviolet diode array (UV-DAD) and electrospray ionization mass spectrometry (ESI-MS) detection by De Orsi, et. al. [22] was developed for the determination of minoxidil, progesterone, estrone, spironolactone, canrenone, hydrocortisone and triamcinolone acetonide in cosmetic products. It was used the chromatographic parameters based on Zorbax SB-CN (250mm×4.6mm; 5_m) column, at a flow rate of 1.2 mL min^{-1}, column temperature of 30°C, three different detection wavelengths (230, 254 and 280 nm) and mobile phase consisted of (A) water (0.1% TFA) and (B) acetonitrile programmed as follows: 90% A for 1 min, decreased to 10% in 40 min, then increased again to 90% A in 10min. Progesterone in aqueous receiving medium, following *in vitro* skin permeation studies had been analyzed using 5 jim LichroCART® RP- 18 column (125 x 4 mm i.d.), after extraction with chloroform, mobile phase used methanol and water (70:30) at a flow rate of 1 mL min^{-1}, detection at 254 nm at room temperature /23/.

This paper describes a reversed-phase HPLC-DAD method for determination of progesterone in pure forms and in capsule form with minor sample treatment. The developed and validated RP-HPLC-DAD method was proposed as a suitable method for analysis of progesterone in capsules. A mixture acetonitrile- Milli-Q grade water (70:30, v:v) at flow rate of 1 mL min^{-1}, C18 column, injection volume of 10 μL, ambient temperature and a UV detection operated at 240 nm was found to be an appropriate mobile phase allowing adequate and rapid separation of analyte (retention time 5.24 min). The total run time for an assay was approximately 6 min. As shown in Figure 2, the substances were eluted forming well-shaped, symmetrical single peak, well separated from the solvent front. Compared with the HPLC methods reported in literature, the developed HPLC-DAD method allows analysis of progesterone in capsule in a shorter time period /13, 21-24/

(within 5.24 min), with better precision and higher sensitivity [22], and moreover it needs minor amounts of organic solvents /25/. The validation studies showed good recovery, precision and accuracy. These features have provided great advantages in terms of material and time.

5. CONCLUSION

The RP-HPLC-DAD method for determination of progesterone in capsules was completely validated by using sensitivity, stability, specificity, linearity, accuracy and precision parameters. The proposed method has high recovery and excellent reproducibility. For these reasons, it can be used in routine quality control measurement of progesterone.

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