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NEW LC/MS METHOD FOR DETERMINATION OF PROGESTERONE IN HUMAN PLASMA FOR THERAPEUTIC DRUG MONITORING IN PREGNANCY AND GYNECOLOGICAL DISORDERS

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ABSTRACT. A new simple, sensitive and selective liquid chromatography coupled with mass spectrometry (LC/MS) method for quantification of progesterone in human plasma was validated. The analyte was eluted in 1.9 minutes on a reversed phase column (Zorbax SB-C18, 100 mm x 3.0 mm I.D., 3.5 µm) under isocratic conditions using a mobile phase of a 20:80 (v/v) mixture of formic acid 0.1% (v/v) and methanol. The flow rate was 1 ml/min at the column temperature of 45 ºC. The detection of the analyte was in MS/MS mode using an atmospheric pressure chemical ionization source (APCI+, m/z 315.2 → m/z 279.2). The sample preparation was very simple and rapid and consisted in plasma protein precipitation from 0.2 ml plasma using 0.5 ml methanol. Calibration curves were generated over the range of 0.8-80 ng/ml with values for coefficient of determination greater than 0.995 and by using a weighted ($1/y^2$) linear regression. The values of precision (coefficient of variation %) and accuracy (relative error %) were less than 9.4% and 14.2%, respectively, both for within- and between-run analysis. The mean recovery of the analyte was 98.6%. The developed LC/MS/MS method could be applied for determination of progesterone in human plasma for therapeutic drug monitoring in pregnancy and gynecological disorders.

Keywords: *progesterone, human plasma, LC/MS/MS, method validation*

INTRODUCTION

Progesterone (PRG) is a steroid, secreted in large amounts by the corpus luteum and the placenta. It is an important intermediate in steroid biosynthesis in all tissues that secrete steroid hormones and small amounts enter the circulation from the adrenal cortex. It plays a key role in the female menstrual cycle (mainly produced after ovulation) and during pregnancy, when its production causes suppression of further ovulation and provides the correct environment for the developing embryo [1].

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The analysis of steroid hormones in biological samples can be employed as a diagnostic tool in diseases promoted by disorders in the steroids profile. Progesterone is suitable to be monitored during treatment of infertility. Exogenous progestogens are administered in hormone replacement therapy and the modern, accurate and succesfully treatment involves hormon plasma level monitoring. This kind of therapeutic strategy has the problems of low concentration of steroids in plasma and the complexity of sample matrix, and demands for the development of highly selective and sensitive analysis methods. Progress in instrumental analytical chemistry and robust extraction techniques have enabled the detection of more compounds at lower concentrations, contributing to the success of different kind of therapies.

Liquid Chromatography coupled with Mass Spectrometry (LC–MS) has been commonly selected for the analysis of the steroids due to its advantages of high sensitivity and selectivity of MS and allows a very short analysis run-time. Many applications have been reported, regarding steroid hormons determinations, including, determinations in water [2-9], tissues [10-12], food [13], cosmetics [14] and only a few in urine, serum or blood [15-17].

In the present study, we attempted to develop a fast HPLC/MS/MS method able to quantify PRG in human plasma after a simple protein precipitation for both physiological and therapy levels monitoring of PRG. Then, the developed method was applied to monitor PRG level in pregnant women or with gynecological disorders under or without PRG treatment.

RESULTS AND DISCUSSION

No significant interference at the retention time of PRG (1.4 min) (Figure 1) was observed in different human male plasma blank samples chromatograms due to the specificity of selected signals (Figure 2).

Two ionization sources were tested, atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI), respectively. Finally, the APCI source was used due to the absence of pH-ionizable groups on progesterone molecule which determined a lower signal when electrospray ionization mode was applied. Different organic solvents were used for mobile phase: acetonitrile and methanol. At the same retention time for the analyte (obtained by using 72% acetonitrile with 28% formic acid 0.1% or 80% methanol with 20% formic acid 0.1%), the signal intensity of PRG was about 3 times higher in case of methanol and for this reason, this was selected for further investigation.

All the studied literature papers propose m/z 109.1 and/or 97.1 as daughter ions for monitoring. Our experiments demonstrated that, in this conditions, later eluting compounds interfere the determination (Figure 3, upper image), so a supplementary wash period is needed which extends the analysis with another six minutes. The selected monitoring ion m/z 279.2 alows a specific and sensitivie analysis in a very short run-time of 2 minutes (Figure 2 and Figure 3 lower image).

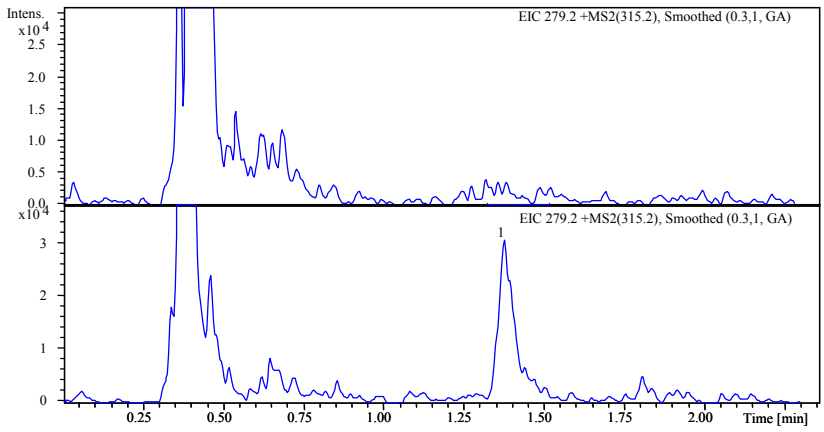

Figure 1. Chromatograms of a blank human male plasma (upper image) and a plasma standard sample of 0.8 ng/ml PRG (RT 1.4 min)(lower image)

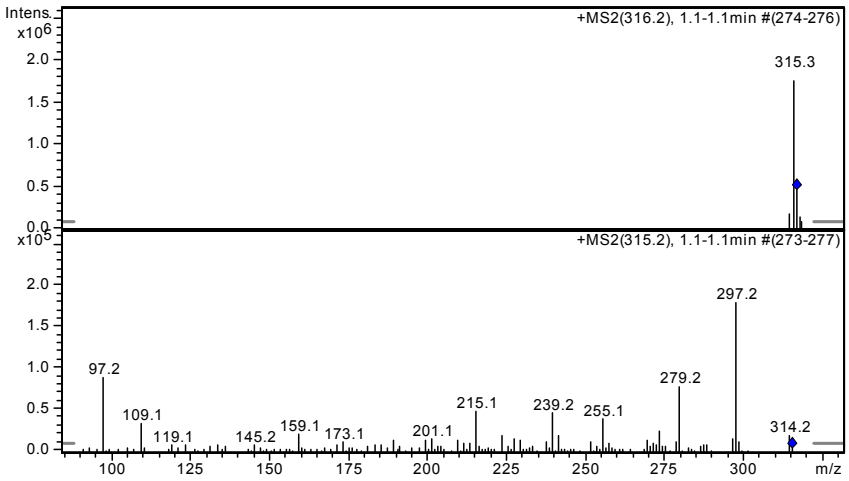

Figure 2. Mass spectra of progesterone: MS/MS non-reactive spectrum - isolation (upper image); MS/MS reactive spectrum - fragmentation (lower image)

The applied calibration curve model proved to be linear over the concentration range 0.8 - 80 ng/ml PRG, with a determination coefficient greater than 0.995. The mean calibration curve, y = a (±SD) x + b (±SD) with SD standard deviation, was: y = 18097.2 (±1850.7) x – 1018.4 (±538.3), N = 8 calibration points, n = 5 determinations for each calibration point. The residuals had no tendency of variation with concentration and were between ±15% values.

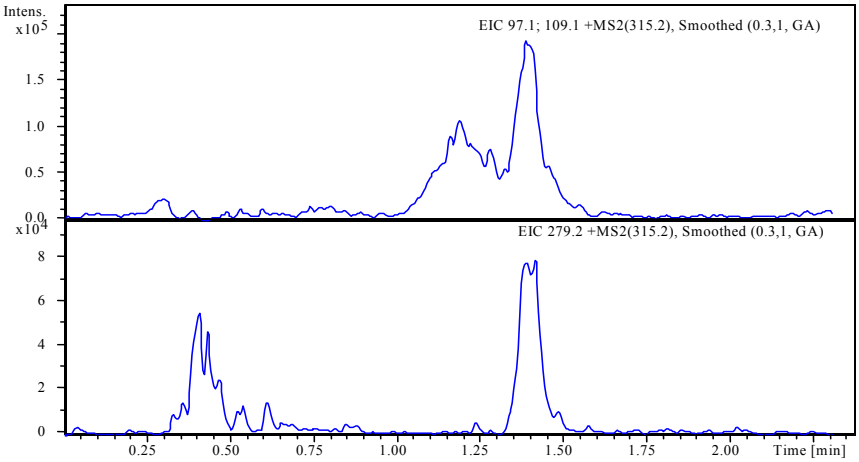

Figure 3. Chromatograms of the sample plasma with PRG (RT 1.4 min), different detection channels: transition 315.2>(97.1 + 109.1) (upper image) and transition 315.2> 279.2 (lower image)

The method had within- and between-run accuracy and precision (Tables 1 and 2), in agreement to international regulations regarding bioanalytical methods validation [18-20]. The lower limit of quantification was established at 0.8 ng/ml PRG, with accuracy and precision less than 20% (Tables 1 and 2).

The recovery was consistent and reproducible (Table 1 and 2).

The analyte proved its stability after sample preparation for at least 12 hours, the Bias% of found concentration being less than 15%, the maximum accepted value for method's accuracy.

Table 1. Within-run precision, accuracy and recovery for PRG (n = 5)

Nominal concentration ng/ml	Measured concentration ng/ml (± D.S.)		Precision %	Accuracy %	Recovery % (± D.S.)	
0.80	0.88	0.04	4.5	10.0	102.0	4.5
2.40	2.35	0.19	8.0	-2.0	101.6	4.8
16.00	15.87	1.50	9.4	-0.8	94.0	5.5
32.00	34.47	1.69	4.9	7.7	94.6	7.4

Table 2. Between-run precision, accuracy and recovery for PRG (n = 5)

Nominal concentration ng/ml	Measured concentration ng/ml (± D.S.)		Precision %	Accuracy %	Recovery % (± D.S.)	
0.80	0.92	0.07	7.4	14.2	98.6	8.4
2.40	2.54	0.05	2.1	5.7	100.8	3.7
16.00	16.61	0.76	4.5	3.8	98.8	7.6
32.00	33.62	2.50	7.4	5.1	98.2	7.6

In comparison with the studied chromatographic – mass spectrometry methods about PRG determination in human plasma, the sensitivity of the proposed method (LLOQ of 0.8 ng/ml PRG in plasma) is better [16,17]. But the main advantage, except the short run-time of 2 minutes, is the sample preparation by protein precipitation and besides its simplicity, that sample treatment allows obtaining a very good recovery of analyte. The current LC/MS method does not intend to be a competitor or to replace the immunoassays methods, and is focused as an analytical tool for therapeutic drug monitoring of progesterone. In Table 3 a critical comparison between determination of progesterone by LC/MS and immunoassay, focused on the main advantages of the former technique, is given.

Table 3. Comparison between determination of progesterone by LC/MS and immunoassay

Parameter	Current LC/MS method	Immunoassay methods EIA, ELISA [18]	Observations
Selectivity	Highly selective	Less selective, possible overestimation due interference of compounds with very similar structure (cross-reaction)	LC/MS method is more selective than immunoassays methods
Sensitivity	0.8 ng/ml	Depends on method/ technique, usually between 0.02-0.2 ng/ml	Although immunoassay methods are more sensitive, the aim of our LC/MS method is therapeutic drug monitoring, and the expected levels of progesterone are in this case more than 10 ng/ml
Upper limit of quantification (ULOQ)	80 ng/ml	Depends on method / technique, usually between 5-50 ng/ml	If concentration is above ULOQ, sample dilution is required and re-analysis, increasing the total analysis time and costs
Matrix effects or interferences	Less susceptible to matrix effects	Highly susceptible to matrix effects due inter- and intra-individual differences in qualitative and quantitative serum composition; internal control required	LC/MS method is more robust due relatively less matrix effects
Analysis time / throughput	Less than 10 min per sample, including sample preparation and analysis	Usually 60-120 min per sample	LC/MS is high-throughput compared with immunoassays methods
Sample volume required	0.2 ml plasma	0.02-0.2 ml serum	Immunoassays methods usually require a smaller sample volume in comparison with LC/MS method
Reagents preparation and costs	In house-preparation and low cost of reagents	Special designed kits of reagents, about 10-50 folds higher costs as in case of LC/MS	LC/MS method is much cheaper regarding reagents costs compared with immunoassays methods
Shelf-life of reagents	The reagent is prepared in house as needed, from components with shelf life more than 3 years	Limited shelf-life of reagent kits, typically 3-18 months in case of un-opened recipient, less than 3 month after opening the reagent bottle	No problem with shelf-life of reagents in case of LC/MS method

Regarding method's applicability, as it can be seen from Table 4, the proposed analytical method demonstrated the increasing of the level of progesterone in human plasma in the luteal phase of menstrual cycle and it's decreasing in menopause. During the pregnancy and also during the treatments with Utrogestan 100 mg PRG, the level of progesterone in human plasma is increased.

Table 4. Levels of PRG in pregnant or in women with gynecological disorders with or without PRG treatment

Subject	Diagnostic	Observations	Treatment with PRG	PRG level found (ng/ml)
S01	Pelvic inflamatory disease	Age 22 years, The luteal phase of teh menstrual cycle, day 22	No	5.69
S02	Endocervical polyp	Age 52 years, Menopause from 5 years	No	<LOQ
S03	Pregnancy, Nausea and vomiting	Age 29 years, Pregnancy 6-WG	No	20.64
S04	Pregnancy Threatened abortion	Age 28 years, Pregnancy 22- WG	No	43.43
S05	Pregnancy Placenta previa with bleeding	Age 25 years, Pregnancy 25- WG	No	44.56
S06	Pregnancy Mild preeclamsia, Preterm birth	Age 22 years, Pregnancy 34- WG	No	85.24
S07	Pregnancy Threatened abortion	Age 31 years, Pregnancy 12- WG	Utrogestan, 2x1 cps/day, 3 weeks	23.77
S08	Pregnancy Threatened abortion	Age 26 years, Pregnancy 22- WG	Utrogestan, 2x1 cps/day, 14 weeks	32.15
S09	Pregnancy Threatened abortion	Age 28 years, Pregnancy 8- WG	Utrogestan, 2x1 cps/day, 2 weeks	20.20
S10	Pregnancy Cervical incompetence with cerclaj Preterm birth	Age 27 years, Pregnancy 32- WG	Utrogestan, 4x1 cps/day, 24 weeks	50.98
S11	Pregnancy Cervical incompetencewith cerclaj Threatened abortion	Age 34 years, Pregnancy 13- WG	Utrogestan, 2x1 cps/day, 10 days	25.5
S12	Abnormal uterine bleeding	Age 43 years	Utrogestan, 3x1 cps/day, 3 days	12.34

CONCLUSIONS

The proposed method provides accuracy and precision for quantitative determination of progesterone in human plasma for therapy of pregnant women or with gynecological disorders. The simple sample preparation by protein precipitation, the selected signal for monitoring and the short run-time allow a specific and efficient analysis of a large number of plasma samples, making the method more productive and thus more cost effective.

The proposed method allows evaluation of progesterone in human plasma in different phases of the menstrual cycle, menopause and also in the response of the progesterone replacement therapy, together to evaluation of pregnancies prognosis and the efficiency of Utrogestan therapy.

EXPERIMENTAL SECTION

Reagents

Progesterone (PRG) was reference standards from Sigma-Aldrich (St. Louis, MO, SUA). Methanol and formic acid were Merck products (Merck KgaA, Darmstadt, Germany). Distilled, deionised water was produced by a Direct Q-5 Millipore (Millipore SA, Molsheim, France) water system. The human blank plasma was obtained from male volunteers.

Standard solutions

A stock solution of PRG with concentration of 2 mg/ml was prepared by dissolving appropriate quantities of reference substance (weighed on an Analytical Plus balance from Ohaus, USA) in 10 ml methanol. A working solution of 8000 ng/ml was then obtained by diluting specific volume of stock solution with plasma and it was further diluted with plasma to 80 ng/ml. Then these working solutions were used to spike different volumes of plasma blank, providing finally eight plasma standards with the concentrations ranged between 0.8 and 80 ng/ml. Accuracy and precision of the method was verified using plasma standards with concentrations 0.8, 2.4, 16 and 32 ng/ml PRG. Quality control samples (QC) at 2.4 (QCA), 16 (QCB) and 32 (QCC) ng/ml analyte will be used during clinical samples analysis.

Chromatographic and mass spectrometry systems and conditions

The HPLC system was an 1100 series model (Agilent Technologies) consisted of a binary pump, an in-line degasser, an autosampler, a column thermostat, and an Ion Trap SL mass spectrometer detector (Brucker Daltonics GmbH, Germany). Chromatograms were processed using QuantAnalysis software. The detection of the analyte was in MS/MS mode using an atmospheric pressure chemical ionization source (APCI), positive ionization, by monitoring the transition m/z 315.2 → m/z 279.2). Other apparatus parameters: capillary 3500 V, vaporizer temperature 450 °C, nebulizer 60 psi, dry gas temperature 300 °C, dry gas flow 7.00 L/min. Chromatographic separation was performed at 45ºC on a Zorbax SB-C18 100 x 3 mm, 3.5 µm column (Agilent Technologies), protected by an in-line filter.

Mobile phase

The mobile phase consisted of a mixture of formic acid 0.1% (V/V) and methanol (20:80 v/v), each component being degassed, before elution, for 10 minutes in an Elma Transsonic 700/H (Singen, Germany) ultrasonic bath. The pump delivered the mobile phase at 1 ml/min.

Sample preparation

Standard and test plasma samples were prepared as follows in order to be chromatographically analyzed. In an Eppendorf tube, to 0.2 ml plasma, 0.5 ml methanol was added. The tube was vortex-mixed for 10 seconds and then centrifuged for 3 minutes at 10000 rpm. A volume of 150 µl supernatant was transferred in an autosampler vial and 30 µl were injected into the HPLC system.

Analytical performance of the method

The bio-analytical methods for utilization in pharmacokinetics-bioavailability studies and therapeutic drug monitoring are conducted in concordance with FDA, EMEA and laboratory's SOP regulations [19-26].

As a first step for the analytical performance determination of the method, specificity was verified using six different plasma blanks obtained from healthy human male volunteers. The progesterone level in men plasma not exceeds 0.1-0.4 ng/ml, however plasma with no detected PRG was used in order to obtain calibrators and quality control samples.

The concentration of analytes was determined automatically by the instrument data system using the external standard method. Calibration was performed using singlicate calibration standards on five different occasions. The calibration curve model was determined by the least squares analysis. The applied calibration model was a linear one: $y = ax + b$, $1/y^2$ weight, where y – peak area and x – concentration. Distribution of the residuals (% difference of the back-calculated concentration from the nominal concentration) was investigated. The calibration model was accepted, if the residuals were within ±20% at the lower limit of quantification (LLOQ) and within ±15% at all other calibration levels and at least 2/3 of the standards met this criterion, including highest and lowest calibration levels.

The lower limit of quantification was established as the lowest calibration standard with an accuracy and precision less than 20%.

The within- and between-run precision (expressed as coefficient of variation, CV%) and accuracy (expressed as relative difference between obtained and theoretical concentration, Bias%) of the assay procedure were determined by analysis on the same day of five different samples at each of the lower (2.4 ng/ml), medium (16 ng/ml), and higher (32 ng/ml) levels of the considered concentration range and one different sample of each on five different occasions, respectively.

The relative recoveries at each of the previously three levels of concentration and limit of quantification were measured by comparing the response of the treated plasma standards with the response of standards in solution with the same concentration of analytes as the prepared plasma sample.

The post-preparative stability (PPS) in the autosampler of the analytes in human plasma was investigated at lower (2.4 ng/ml) and higher concentration (32 ng/ml) for 12 hours, the expected longest storage times of the samples in autosampler before injection. The requirement for stable analytes was that the difference between mean concentrations of the tested samples in various conditions and nominal concentrations had to be in ±15% range.

Clinical application

The developed method was verified by analyzing different plasma samples obtained from pregnant women or with gynecological disorders, under treatment with vaginal capsules of PRG (Utrogestan 100 mg vaginal capsules) or without treatment.

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