Application Note

Clinical Research

Progesterone Metabolism in Serum

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Abstract

Progesterone and its metabolites play an important role in the female body. Therefore, it is often used in hormone replacement therapies. However, current research has shown that progestins—synthetic progesterone—can play a role in various diseases, especially breast cancer.

The LC/MS/MS method presented here, which comprises the Agilent 1290 Infinity II LC and 6495 triple quadrupole LC/MS, is for the analysis of progesterone and relevant, dependent hormones. These are progesterone, 3α-, 5α-, and 20α-dihydroprogesterone as well as allopregnanolone, pregnanolone, and deoxycorticosterone.

Introduction

In addition to estrogen, progesterone and its metabolites play a role in the development of breast cancer, increasing the risk of its development.[1,2] The examination of breast tissue and tumor breast tissue has shown the involvement of three progesterone metabolites: 3α-, 5α-, and 20α-dihydroprogesterone.[1,3] In addition to progesterone, 5α-dihydroprogesterone has a breast cancer-promoting effect, while the other two metabolites, 3α- and 20α-, inhibit the development of breast cancer.[4] Up to now, progestins and progesterone have been widely used in hormone-replacement therapy, but their effects can be varied due to their different chemical properties, triggering negative side-effects.[5] The LC/MS/MS method developed here is intended as a means of profiling hormone levels from serum in men and women.

Experimental

LC configuration and parameters

Configuration	
Agilent 1290 Infinity II high-speed pump (G4220A)	
Agilent 1290 Infinity II multisampler (G7167B) with sample thermostat (G7167-60101)	
Agilent 1290 Infinity II thermostatted column compartment (G7116B)	
Needle Wash	50/50 water/acetonitrile
Autosampler Temperature	10 °C
Injection Volume	10 µL
Analytical Column	BEH C_{18} 100 × 2.1 mm, 1.7 µm (Waters Corporation)
Column Temperature	30 °C
Mobile Phase A	Water with 0.1% formic acid
Mobile Phase B	Acetonitrile with 0.1% formic acid
Flow Rate	300 µL/min
Gradient	Time (min) %B 0.00 20 1.50 55 5.50 80 5.60 95 6.60 95 6.70 20 8.00 20
Stop Time	8 minutes

LC/TQ mass spectrometer configuration and parameters

Configuration	
Agilent 6495 triple quadrupole LC/MS (G6495B)	
Ionization Mode	Positive
Drying Gas Temperature	210 °C
Drying Gas Flow	11 L/min
Nebulizer Pressure	35 psi
Sheath Gas Temperature	250 °C
Sheath Gas Flow	12 L/min
Nozzle Voltage	500 V
Capillary Voltage	3,000 V
Delta EMV	200 V

Chemicals and reagents

Formic Acid ≥99%, LC/MS Grade	VWR (Darmstadt, Germany)
Acetonitrile	Biosolve (Valkenswaard, The Netherlands)
Hydroxylamine Solution (50 wt. % H_2O)	Sigma-Aldrich (Darmstadt, Germany)
Sodium hydroxide Solution (50%)	Sigma-Aldrich (Darmstadt, Germany)
tert-Butyl methyl ether	Merck (Darmstadt, Germany)
4-Pregnen-3α-ol-20-one	Steraloids (Newport, RI, USA)
5α-Pregnan-3,20-dione	Steraloids (Newport, RI, USA)
4-Pregnen-20α-ol-3-one	Steraloids (Newport, RI, USA)
5α-Pregnan-3α-ol-20-one	Sigma-Aldrich (Darmstadt, Germany)
21-Hydroxyprogesterone	Sigma-Aldrich (Darmstadt, Germany)
5β-Pregnan-3α-ol-20-one	Sigma-Aldrich (Darmstadt, Germany)
Progesterone	Sigma-Aldrich (Darmstadt, Germany)
Progesterone-2,3,4-$^{13}C_3$	Sigma-Aldrich (Darmstadt, Germany)
Phosphate-Buffered Saline (PBS)	Sigma-Aldrich (Darmstadt, Germany)
Bovine Serum Albumin (BSA)	Sigma-Aldrich (Darmstadt, Germany)

Standards and curve preparation

All analytes were dissolved in acetonitrile. A mixture with a concentration of 1 mg/L in acetonitrile was then prepared. Seven standard concentrations were prepared to cover a working range between 5 ng/L and 1 µg/L. The internal standards were dissolved in acetonitrile and used at a concentration of 1 µg/L. Quality controls were prepared in a 0.1% BSA solution in PBS, as no commercially available controls were available. The concentrations of the controls cover the lower and middle range of the standard series.

Sample preparation

Standard, quality control, or serum sample (400 µL) was aliquoted, followed by 50 µL addition of internal standard. For the extraction of the progesterone metabolites, 5 mL MTBE was added to the sample. Afterwards, the sample was vortexed and centrifuged at 4,700 rpm for 5 minutes.

The organic supernatant was transferred and evaporated at 45 °C under nitrogen. The residue was reconstituted in 600 µL of 1 M hydroxylamine solution (0.5% NaOH) and vortexed. The derivatization reaction continued for 15 minutes at 90 °C. During the reaction, the solution was shaken at 500 rpm. After cooling, 5 mL of MTBE was added, and the mixture was vortexed. After centrifugation (5 minutes, 4,700 rpm), the supernatant was removed and evaporated at 45 °C under nitrogen. The residue was then reconstituted with 100 µL water/acetonitrile (70%/30%) and transferred to a sample vial for analysis.

Data analysis

Data acquisition was performed using Agilent MassHunter Acquisition software (B.08.00). Data were analyzed using Agilent MassHunter Quantitative Analysis software (B.08.00). All analytes were corrected with the internal standard used (progesterone-2,3,4-$^{13}C_3$) to exclude matrix effects.

Results and discussion

The LC/MS/MS method was able to separate all progesterone metabolites, as shown in an example serum sample in Figure 1. The relevant separation of the isomers was also successful.

Table 1. Mass transitions, retention times, and collision energy of the progesterone metabolites investigated.

Analyte	Retention Time (min)	Mass Transitions (Da)	Collision Energy (V)
Deoxycorticosterone	2.9	361.3 → 124.1	33
20α-Dihydroprogesterone	3.8	332.3 → 124.1	33
Progesterone	3.9	345.3 → 124.1	37
Progesterone-2,3,4-$^{13}C_3$	3.9	348.3 → 127.3	29
5α-Dihydroprogesterone	4.1	347.3 → 86.0	33
3α-Dihydroprogesterone	4.4	332.3 → 86.1	33
Pregnanolone	4.6	334.3 → 86.0	41
Allopregnanolone	4.7	334.3 → 86.0	33

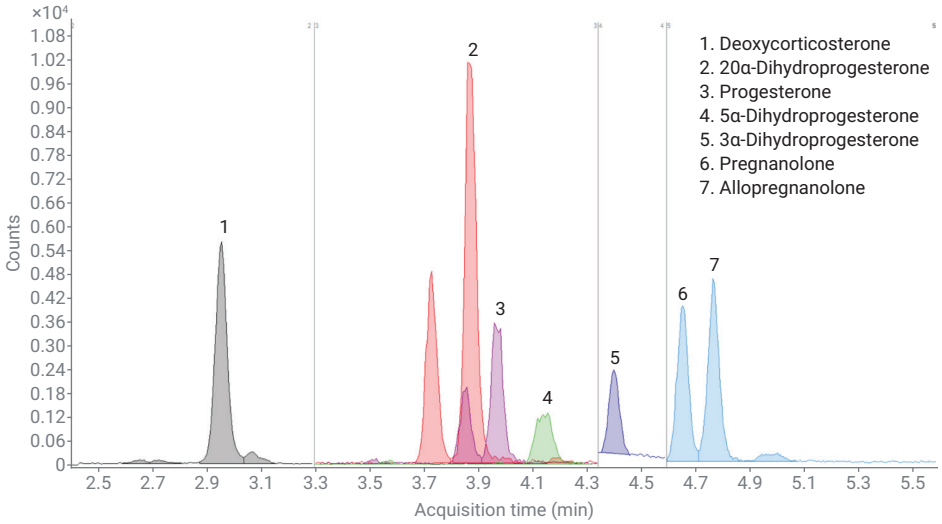

Figure 1. Chromatogram of an example serum sample. The gray lines separate the time windows in which only the MRM transitions of the selected analytes are measured to achieve a better dwell time.

The analytical validation showed satisfactory values in intra- and inter-assay precision, with all values below 10%. Precision data are shown in Table 2, in addition to limit of quantification (LOQ) and linearity data.

To test the LC/MS/MS method, serum samples from men and postmenopausal women were processed and measured. The results for both sexes show similar values for the relevant progesterone-related hormones and the ratio (Table 3).

Table 2. Analytical validation data consisting of inter-assay, intra-assay, limit of quantification (LOQ), and linearity.

Analyte	Intra-Assay (%)	Inter-Assay (%)	LOQ (ng/L)	Linearity (ng/L)
Deoxycorticosterone	4.3	8.6	1.00	5 to 1,000
20α-Dihydroprogesterone	5.6	8.5	4.10	
Progesterone	5.2	9.4	2.48	
5α-Dihydroprogesterone	4.7	7.7	0.80	
3α-Dihydroprogesterone	6.8	5.4	0.80	
Pregenanolone	4.4	8.6	1.00	
Allopregnanolone	6.8	8.6	0.80	

Table 3. Median (ng/L) of relevant progesterone-related hormones and the ratio of 5α- to 3α-dihydroprogesterone by sex.

Analyte	Men (n = 64)		Postmenopausal Women (n = 54)	
	Median	CI 95%	Median	CI 95%
20α-Dihydroprogesterone	58.3	26.8 to 211.4	40.0	8.5 to 127.6
Progesterone	48.2	24.8 to 154.2	27.9	10.5 to 132.6
5α-Dihydroprogesterone	26.4	7.7 to 148.2	16.3	3.1 to 202.4
3α-Dihydroprogesterone	2.0	1.0 to 5.7	1.7	0.9 to 8.4
5α/3α Ratio	13.6	3.4 to 29.9	8.2	2.2 to 68.5

CI = confidence interval

Conclusion

The LC/MS/MS method presented in this application note offers the possibility to reliably measure progesterone and relevant metabolites. The LOQ achieved also allows the measurement of these hormones in men and postmenopausal women, who naturally have lower levels of progesterone. It should be noted that the preparation of the samples is complex and has an influence on the measurement result; therefore, great care is required.

The results of the test measurements for progesterone and 20α-dihydroprogesterone are comparable with the literature: 48.2 to 42 ng/L for men and 27.9 to 34 ng/L for women for progesterone and 58.3 to 39 ng/L and 40 to 40 ng/L for 20α-dihydroprogesterone. Likewise, the ratio between 5α- and 3α-dihydroprogesterone aligns well with literature values (10.9 to 9).[4]

The values for 3α- and 5α-dihydroprogesterone are significantly higher in the literature, which is probably due to the use of another internal standard.[4,6]

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References

1. Trabert, B. *et al.* Progesterone and Breast Cancer. *Endocr. Rev.* **2020**, *41(2)*, 320–344.
2. Trabert, B. *et al.* Association of Circulating Progesterone With Breast Cancer Risk Among Postmenopausal Women. *JAMA Netw, Open* **2020**, *3(4)*, e203645.
3. Wiebe, J. P.; Progesterone Metabolites in Breast Cancer. *Endocr. Rela. Cancer* **2006**, *13(3)*, 717–738.
4. Trabert, B. *et al.* Reproducibility of an Assay to Measure Serum Progesterone Metabolites That May Be Related to Breast Cancer Risk Using Liquid Chromatography-Tandem Mass Spectrometry. *Horm. Mol. Biol. Clin. Investig.* **2015**, *23(3)*, 79–84.
5. Stanczyk, F. Z. *et al.* Progestogens Used in Postmenopausal Hormone Therapy: Differences in their Pharmacological Properties, Intracellular Actions, and Clinical Effects. *Endocr. Rev.* **2013**, *34(2)*, 171–208.
6. Hada, M. *et al.* Relationship of Serum Progesterone and Progesterone Metabolites with Mammographic Breast Density and Terminal Ductal Lobular Unit Involution among Women Undergoing Diagnostic Breast Biopsy. *J. Clin. Med.* **2020**, *9(1)*, 245.

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