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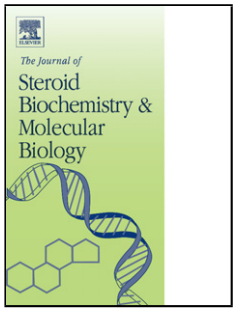

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How high-resolution techniques enable reliable steroid identification and quantification

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Abstract

Due to possible matrix interferences and artefact generation during sample preparation, careful method validation is required for quantitative bioanalytical methods, especially for analytes that are only present in low concentrations. Using the identification and quantification of progesterone metabolite in the urine of newborns as an example, we show how modern high-resolution instruments can be used to verify analyte assignment and avoid pitfalls commonly encountered by the use of low-resolution instruments.

Keywords: Steroids, progesterones, steroidomics, time-of-flight mass spectrometry, two-dimensional gas chromatography, method validation

Introduction

Gas chromatography-mass spectrometry (GC-MS) has been the gold standard for urinary steroid profiling for more than three decades as pioneered by Shackleton.[1,2] The technique has been adopted by numerous labs worldwide and is both robust and sensitive. Yet, adaptation of the method to include additional urinary steroid metabolites is cumbersome and requires careful method validation. Herein, we would like to focus on the possible pitfalls during method development and would like to show how modern bioanalytical technology can help to avoid them.

Chemically speaking, steroids are extremely similar substances that sometimes only differ in their geometry which makes their separation already rather challenging.[3] After separation and prior to mass spectrometric measurement, analytes have to be ionized. For the generation of ions in the GC-MS interface, electron ionization is employed which leads to removal of an electron from the analyte and subsequently charged analytes as required for mass spectrometry where the analytes are detected based on their mass-to-charge ratio (m/z). Due to the energy of the electrons that are colliding with the analytes in the ion source during the ionization reaction, extensive fragmentation of the parent molecule takes place.[4] The fragmentation mechanisms are generally well understood and the fingerprint provided by the spectra can aid in structural analysis and analyte identification. Nonetheless, fragmentation also leads to rather complicated spectra that can impede data analysis (Figure 1). This is the case especially in complex samples where multiple compounds might co-elute from the chromatography column thus leading to mixed spectra. Also, the ions of many compounds exhibit similar mass which cannot be distinguished by low-resolution mass spectrometers as commonly employed in GC-MS instruments. This may lead to incorrect peak assignment and misinterpretation.

We have recently developed a method for the analysis of urinary steroids based on high-resolution GC and MS, namely GCxGC-TOF MS (multidimensional gas chromatography- time of flight mass spectrometry).[5] The improvements compared to conventional GC-MS instruments are multi-fold: first, chromatographic separation of analytes is improved by using a second GC column; second, the exact mass measurements and isotopic pattern analysis using a TOF MS enable reliable analyte identification (Figure 1, B). In addition, GC-MS is usually carried out as a targeted analysis, meaning that the analytes

of interest have to be pre-defined and due to instrumental limitations, usually only the signal for two masses are recorded per analyte. In contrast, in TOF MS complete mass spectra are recorded all the time, thus enabling untargeted analysis. We could show that GCxGC-TOF MS results correlate well with GC-MS thus validating the method originally developed by Shackleton. Furthermore, as GCxGC-TOF MS is more sensitive, it allows detection of urinary steroid metabolites previously not included in our steroid profile panel.

We therefore wanted to include in our high-resolution workflow routine panel the quantification of 22 additional progesterone metabolites in the urine of newborns as reported by Dhayat et al.[6] For that, we re-analyzed 52 samples from the original study to evaluate the performance of GCxGC-TOF MS on these compounds, and to confirm correct analyte assignment. It is noteworthy that interpretation of the original GC-MS data was very cumbersome and some of the reported values were suspiciously high. As an example, the signal recorded for progesterone on a low-resolution GC-MS in both the calibration mixture as well as a patient sample is shown in Figure 2. Whereas peak assignment in the calibration mixture is still straightforward (peak at 17 min), interferences of other metabolites in the mixture are already detectable. In the patient sample, the problem of interference becomes obvious. Over the course of the analysis, numerous compounds with the identical mass are detected, rendering correct peak assignment virtually impossible.

In contrast, measurement of the same sample on a high-resolution instrument is shown in Figure 3. The second GC dimension enables better separation of the analytes, thus improving identification and quantitation. Also, the complexity of clinical samples becomes apparent, with more than 150 steroids or related compounds (e.g. bile acids) being detected. It is obvious that assignment purely based on retention time and single MS signals as in low-resolution instruments becomes problematic, especially for low-abundance compounds.

One of the metabolites which was erroneously quantified in the original study is 17α-hydroxyprogesterone. For quantitation, the ion with m/z of 460 was used based on reference measurements of an authentic standard. In Figure 4, we show how the misassignment would have been avoided using a high-resolution mass spectrometer. On top, we show the mass spectra of the peaks from the reference measurement of the calibration standard next to the peak, which was originally assigned to the compound in a urine sample. Using the accurate mass measurement, the authenticity is confirmed by both calculating the molecular formula ($C_{26}H_{44}N_2O_3Si^+$) and theoretical isotopic pattern (indicated with green bars in the figure) based on this formula. The error between

measured and theoretical mass is 4.34 parts per million (ppm); up to 10 ppm are allowed based on the accuracy of the measurement and there is a high isotopic similarity. In contrast, the ion at m/z 460 in the sample shows both high mass error (16.5 ppm) and low isotopic similarity. Actually, it is obvious that the peak at 460.30396 in the sample is actually the M+1 isotope of the peak at 459.30164 which corresponds to an ion with the molecular formula $C_{26}H_{44}N_2O_3Si^+$. Consequently, the signal from a different compound was attributed to 17α-hydroxyprogesterone in the original study. As an additional level of confirmation, not only the accurate mass and isotopic pattern of the target compound are compared but also the complete mass spectrum. By matching the reference spectrum to the sample spectrum, a score for spectral similarity can be calculated for the verification of compound identification. This is again shown for 17α-hydroxyprogesterone where reference and sample show very low spectral similarity. For further details on how isotopic pattern and spectral similarity scores are calculated, we kindly refer the interested reader to more specialized literature.[7,8]

We applied this multifactorial identification approach based on high-resolution data to 52 samples from the original study (4 infants, 2 males and 2 females, each 13 time points within the first year of life). As can be seen in Table 1, identification could only be verified for approximately a quarter of all analytes and time points and 10 progesterone metabolites were not detected at all (Table 2). Criteria for positive identification was next to correct retention time in both dimensions, accurate mass, isotopic pattern distribution, the detection of this metabolite in at least 50% of the samples at the given time point. In addition to issues with identification, problems with quantification in the low-resolution data also become evident. In contrast to the original study where rather high concentration of some metabolites were reported (> 10000 ug/mmol creatinine in some cases), the values based on high-resolution data are on average significantly lower (up to a factor of 300) for all but two metabolites.

Conclusion

Due to the complexity of matrixes like urine and problems of separating and identifying steroids that display very high chemical similarity, method development for the quantification of low abundance

steroids is highly challenging. Herein we show that high resolution techniques, which have become more widely available since the rise of "-omics" are extremely beneficial for this purpose. Traditional, low-resolution techniques still have their merits, especially when it comes to ease-of-use and costs and will continue to find application. We suggest that initial method development for challenging analytes as described in this paper should be performed on high-resolution instruments, then transferred to low-resolution instruments and re-validated for routine clinical applications. This recommendation results from own errors with low resolution analysis of baby urinary steroid metabolites[6], detected by re-analysis with high-resolution techniques (see Tables 1 and 2).

Methods

Progesterone metabolites standards as well as the two internal standards stigmasterol and medroxyprogesterone (all ≥ 99% purity), were purchased from Steraloids (Newport RI, USA). All analytes are listed in Tables 1 and 2. Methanol, hexamethyldisilazane and cyclohexane (HPLC-MS grade), sodium acetate and pyridine were purchased from Merck (Switzerland). Sep-Pak C18 cartridges (Waters, Switzerland) as well as Lipidex 5000 (Perkin Elmer, Waltham MA, USA) were used for sample clean-up procedures. Powdered sulfatase, β-glucoronidase (both Sigma-Aldrich, Switzerland) and β-glucoronidase/arylsulfatase (Roche Diagnostics, Switzerland) all originated from Helix pomatia were used for hydrolysis. For derivatization of steroids methoxamine (MOX) and N-trimethylsilylimidazole (TMSI) were purchased from Thermo Fisher Scientific (Switzerland).

Calibration was carried out using a mixture of all 21 progesterone metabolites. Validation of the analytical method with regard to of intra- and inter-day instrument stability, reproducibility, sensitivity, accuracy and recovery rate has been reported elsewhere.[5]

Urine sample preparation was performed as originally described by Shackleton[1] as follows: (i) steroid extraction from urine, (ii) hydrolysis, (iii) second extraction of the steroids, (iv) derivatization and (v) sample clean-up prior to GC-MS and GCxGC-TOF MS analysis.[1,6]

GC-MS analysis was performed by coupling a 7890A gas chromatograph to a 5795C mass spectrometer (both Agilent Technologies, Switzerland). Details of the GC-MS analysis have been previously published.[6]

For GCxGC-TOF MS, all samples were analyzed using a 7890B GC system equipped with a G4513A auto-sampler (Agilent Technologies, Switzerland) and coupled to a high-resolution EI-TOF mass spectrometer (Tofwerk, Switzerland). A Zoex double focusing loop modulator (ZX2 Thermal Modulator, Zoex Corp., USA) was mounted in the GC oven between the first (GC1) and second (GC2) column. The eluate of the first column was trapped in the modulation loop with a cold jet of nitrogen (-80 °C). A hot jet of nitrogen was then used for rapid desorption at a modulation period of 6 s. For GC1, a 15 m x0.25

mm i.d. x 0.25 µm RXI-1ms column (crossbond dimethyl polysiloxane, Restek Corporation, USA), for GC2 a 2 m x 0.1 mm i.d. x 0.1 µm BPX50 column (50% phenyl polysilphenylene-siloxane, SGE, USA) was used. Of GC2, 1 m was utilized for the modulation loop. GC oven temperature was held at 50°C for 1 min followed by a first ramp to 220°C at a rate of 30°C/min and a second ramp to 300°C at a rate of 2°C/min and then held at 320°C for 5 min. For the hot jet, an initial temperature of 120°C for 1 min was followed by a first ramp to 240°C at a rate of 30°C/min and a second ramp to 320°C at a rate of 2.5°C/min before returning to 120°C. A constant flow of helium at a rate of 0.8 ml/min was used as carrier gas with an initial head pressure of 1.5 bar. 1 ul of each sample was injected in a split/splitless inlet held at 280°C in pulsed splitless mode. The GC2 column was directly coupled to the MS using a feedthrough block held at 275°C. The ion source temperature was set to 280°C. MS analyses were conducted at 100 Hz in a mass range of 45-670 Th. Electron ionization was performed at an electron energy of 70 eV. The mass spectrum was recalibrated at the beginning of each modulation period using pentafluorophenol (PFP) as an internal standard. Data analysis was performed using GC Image software (Zoex Corp.) version R2.6b3-HRMS and Tofware (Tofwerk) version 2.5.10.

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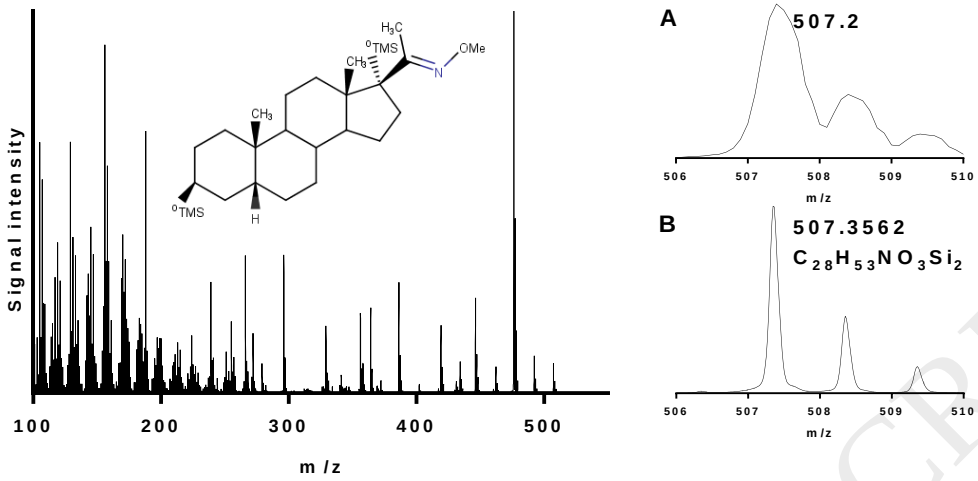

Figure 1. Electron ionization mass spectrum of 17α-hydroxypregnanolone demonstrating the complexity due to extensive fragmentation of the analyte in the ion source (TMS = trimethylsilyl). Insert A shows the peak of the intact molecule measured on a low-resolution MS, insert B measured on a high-resolution MS. The high-resolution instruments allows determination of elemental composition based on accurate mass analysis and in combination with the isotopic pattern enables reliable analyte identification.

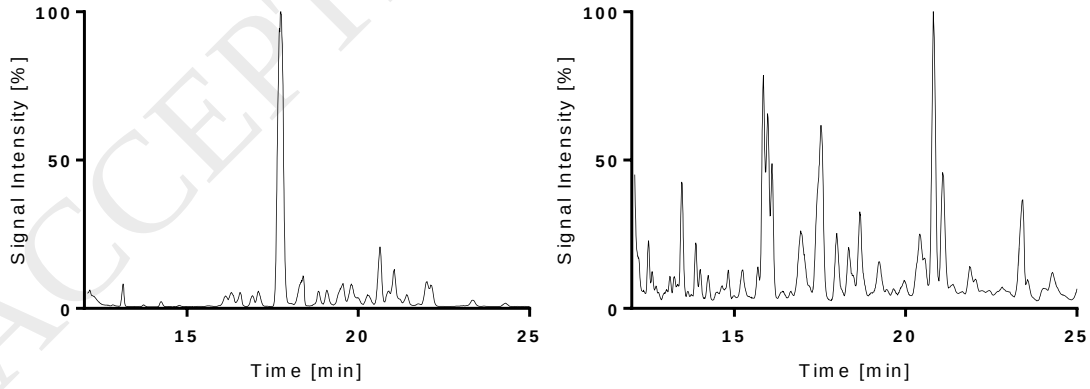

Figure 2. GC-MS chromatograms recorded on a low-resolution instrument. Left: Selected ion monitoring (SIM) of m/z 341 for the detection of progesterone in a mixture of 21 progesterone metabolites. Right: SIM of m/z 341 in an infant urine sample.

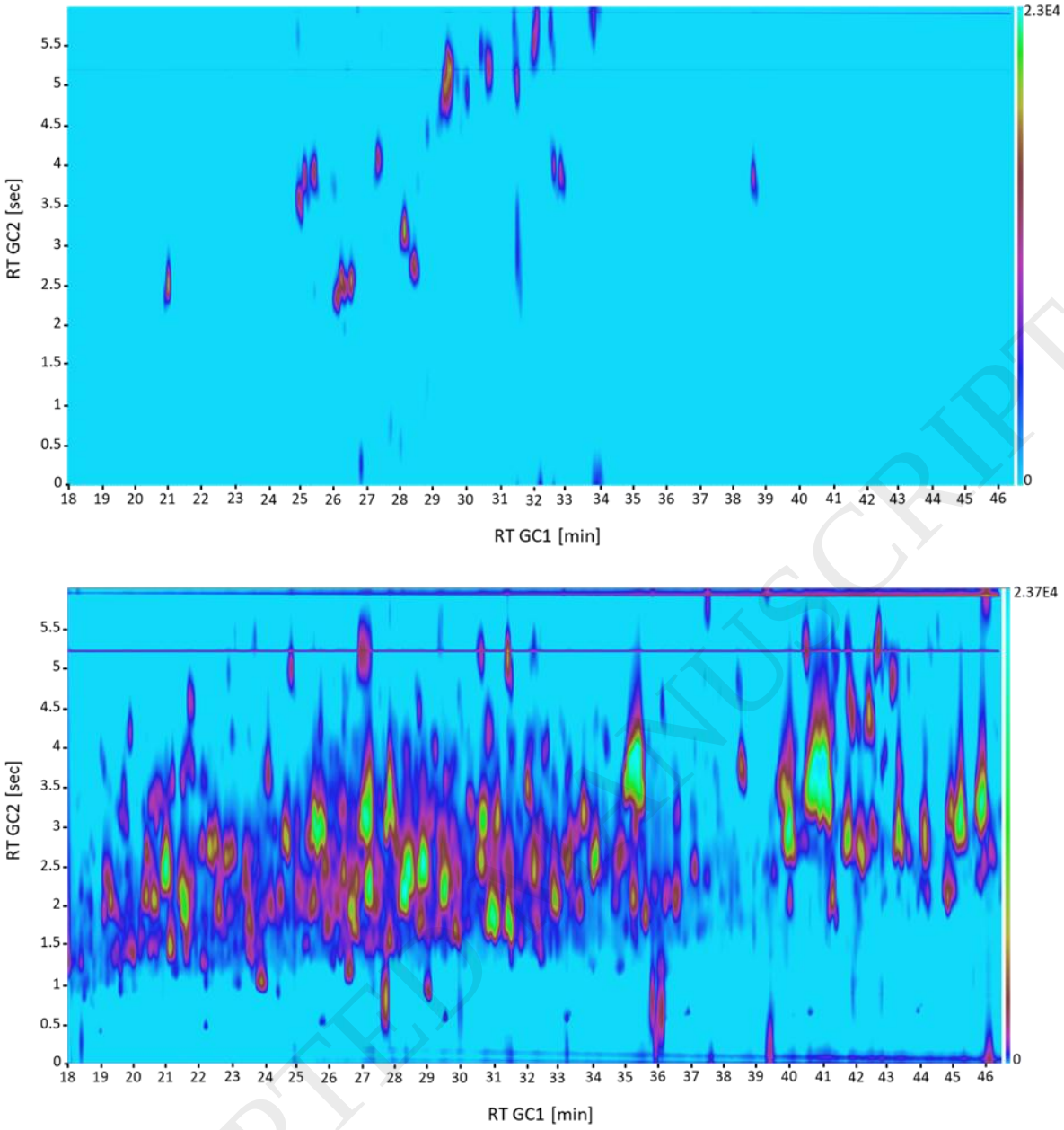

Figure 3. High-resolution GCxGC-TOF MS for steroid analysis. Top: mixture of 21 progesterone metabolite standards. Bottom: infant urine sample. Each blob corresponds to a steroid or related substance (> 150 detected).

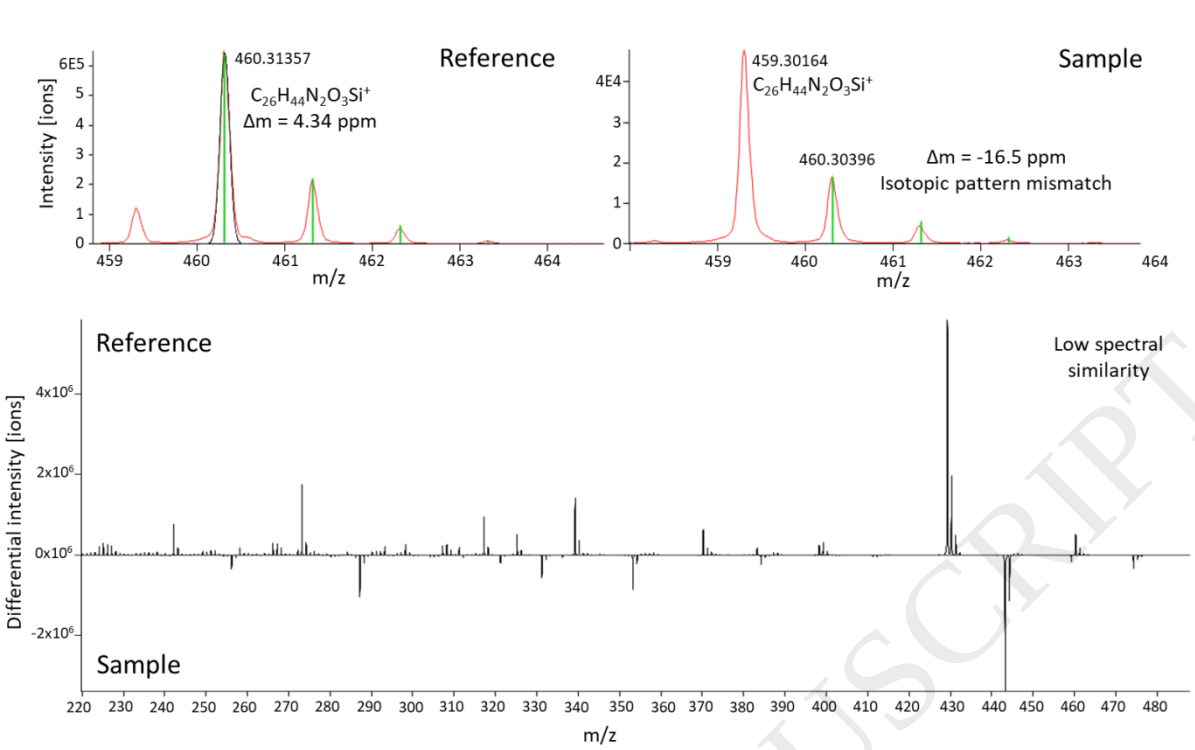

Figure 4. Examples for using accurate mass and isotopic similarity matching (top) as well as spectral similarity matching (bottom) for compound verification in complex matrices. Spectra for 17α-hydroxyprogesterone are shown, theoretical isotopic patterns are indicated with green bars.

Table 1. Results of progesterone metabolite analysis by GCxGC-TOF MS for male and female infants (n=2 each). Average results are shown as absolute values (ug/mmol creatinine) for different time points during the first year of life (W = week). If no value is given, the compound was not detected.

Metabolite		Time Point												
		W1	**W3**	**W5**	**W7**	**W9**	**W11**	**W13**	**W17**	**W21**	**W25**	**W33**	**W41**	**W49**
11a-OH-progesterone	m	221.6	133.5	92.3	62.9	37.2	31.4	11.2	5.0	4.1	9.8	1.5	0.7	1.0
	f	372.3	222.5	184.4	59.3	38.6	19.2	13.8	6.6	3.5	3.2	3.0	1.0	0.7
17a20a-DH-progesterone	m	4.2	2.8	6.1	3.8	5.3	4.2	3.6	2.1	2.1	4.1	1.4	0.9	0.9
	f	4.9	4.7	5.3	3.5	4.7	3.2	3.7	2.1	1.7	1.6	2.1	1.8	2.4
20a-DH-5a-DH-progesterone	m	1.8	-	-	-	-	-	-	-	-	-	-	-	-
	f	5.8	-	-	-	-	-	-	-	-	-	-	-	1.1
20a-DH-progesterone	m	4.3	0.5	1.1	0.1	0.3	-	-	-	-	-	-	-	-
	f	3.1	1.4	-	-	-	-	-	-	-	-	1.0	-	-
20b-DH-progesterone	m	0.4	0.5	-	-	-	-	-	-	-	-	-	-	-
	f	1.6	1.0	-	-	-	-	-	-	-	-	-	-	0.5
3a5a-TH-progesterone	m	13.5	4.0	1.2	3.7	1.3	2.3	-	-	1.1	3.4	-	0.2	0.2
	f	-	5.0	-	-	2.1	-	-	-	-	1.9	-	-	1.0
3b5a-TH-progesterone	m	1.6	1.4	1.6	0.8	-	3.7	1.3	0.7	1.7	1.4	-	-	-
	f	-	-	-	-	-	-	-	-	-	-	-	-	1.7
6a-OH-3a5b-TH-progesterone	m	65.2	56.0	34.3	18.6	43.2	27.3	18.9	8.4	13.2	26.0	13.5	9.9	16.5
	f	772.4	345.9	616.6	200.3	213.8	69.2	30.6	12.0	7.3	5.8	10.7	6.6	3.7
6a-OH-progesterone	m	2.9	1.7	1.0	0.8	0.7	-	-	-	-	-	-	-	-
	f	5.3	5.5	6.3	1.5	-	-	-	-	-	-	-	-	-
6b-OH-progesterone	m	0.6	0.1	0.4	-	0.2	-	0.9	0.2	0.2	0.0	0.2	0.2	0.1
	f	2.4	1.5	4.1	3.6	1.7	2.5	2.1	0.1	0.1	0.2	-	0.1	0.5

Table 2. List of 12 progesterone metabolites that were reported in the original publication[6] but not detected by high-resolution analysis.

Progesterone Metabolites
11b-OH-progesterone
11-keto-progesterone
17a-OH-progesterone
20a-DH-3a5a-TH-progesterone
20a-DH-3b5a-TH-progesterone
20a-DH-3b5b-TH-progesterone
3a5b-TH-progesterone
3b5b-TH-progesterone
5a-DH-progesterone
5b-DH-progesterone
11-Deoxycorticosterone
Progesterone