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A high resolution LC–MS targeted method for the concomitant analysis of 11 contraceptive progestins and 4 steroids

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ABSTRACT

In the context of hormonal contraception and hormone replacement therapy (HRT), many women are exposed to exogenous hormones. Current use of hormonal contraception with combined ethinyl estradiol and different progestins bestows a breast cancer relative risk (RR) of 1.2- while combined HRT has a RR of 2. Although these exposures present an important public health issue, little is known about the effects of individual progestins on the breast and other tissues. Increasing availability of large scale biobanks, high throughput analyses and data management tools enable ever expanding, sophisticated population studies. In order to address the impact of distinct progestins on various health indicators, it is desirable to accurately quantify progestins in clinical samples.

Here we have developed and validated a high resolution liquid chromatography mass spectrometry (LC–MS) targeted method for the simultaneous quantification of 11 synthetic progestins widely used in oral contraceptives, gestodene, levonorgestrel, etonogestrel, chlormadinone acetate, cyproterone acetate, drospirenone, desacetyl norgestimate, medroxyprogesterone acetate, norethindrone, dienogest, nomegestrol acetate, and 4 endogenous steroid hormones, progesterone, testosterone, androstenedione, and cortisol in blood samples. This highly specific quantitative analysis with high resolution Orbitrap technology detects and quantifies 15 compounds using their internal standard counterparts in a single 12 min LC–MS run. Sensitivity is attained by the use of the instrument in targeted selected ion monitoring mode. Lower limit of quantitation ranges from 2.4 pg/ml for drospirenone to 78.1 pg/ml for chlormadinone acetate. The method provides comprehensive progestin panel measurements with as little as 50 μl of murine or human plasma.

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1. Introduction

Steroid hormones are hydrophobic organic molecules derived from cholesterol. Secreted by the gonads, adrenal cortex and the placenta, they regulate a multitude of biological functions. Gluco- mineralo- and corticosteroids are important for metabolism, water/salt balance and immune function while sex steroids control sexual development and reproductive functions.

Progestins are synthetic steroids widely prescribed to women in the context of hormonal contraception and combined hormone replacement therapy. They can be classified according to their chemical structures as: estranes derived from testosterone: norethindrone (NOR), dienogest (DIE), desacetyl Norgestimate (DAcNRG). Pregnanes derived from 17−OH progesterone: medroxyprogesterone acetate (MPA), chlormadinone acetate (CMA), nomegestrol acetate (NOMAC), cyproterone acetate (CPA). Gonanes derived from testosterone: levonorgestrel (LNG), etonogestrel (ETO), gestodene (GSD) and the spironolactone-derivative drospirenone (DSP) [1].

A meta-analysis of 54 epidemiologic studies has shown that the relative risk (RR) of breast cancer for women taking oral con-

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Table 1
List of analytes and internal standards, and their selected properties: MW, monoisotopic mass (m/z), retention time (t_R), retention time coefficient of variation (CV_{tR}).

Analyte	Abbreviation	MW	Formula	[M+H]+ *m/z*	t_R (min)	CV_{tR} (%)
Androstenedione	A4	286.41	C19H26O2	287.20056	5.35	0.20
Androstenedione-13C	A4-^{13}C	289.39	13C3C16H26O2	290.21062	5.35	0.20
Chlormadinone acetate	CMA	404.93	C23H29ClO4	405.18271	7.26	0.11
Chlormadinone acetate-d6	CMA-d6	410.96	C23H23D6ClO4	411.22037	7.23	0.11
Cortisol	F	362.46	C21H30O5	363.21660	3.54	0.49
Cortisol-d4	F-d4	366.48	C21H26D4O5	367.24171	3.52	0.47
Cyproterone acetate	CPA	416.94	C24H29ClO4	417.18271	6.90	0.20
Cyproterone acetate-d3	CPA-d3	419.96	C24H26D3ClO4	420.20154	6.85	0.27
Desacetyl Norgestimate	DAcNRG	327.46	C21H29NO2	328.22710	6.59	0.12
Desacetyl Norgestimate-d6	DAcNRG-d6	333.5	C21H23D6NO2	334.26476	6.57	0.11
Dienogest	DIE	311.42	C20H25NO2	312.19581	3.79	0.42
Dienogest-d8	DIE-d8	319.47	C20H17D8NO2	320.24602	3.74	0.42
Drospirenone	DSP	366.49	C24H30O3	367.22677	5.55	0.16
Drospirenone-d4	DSP-d4	370.52	C24H26D4O3	371.25188	5.53	0.16
Etonogestrel	ETO	324.46	C22H28O2	325.21621	6.45	0.12
Etonogestrel-d7	ETO-d7	331.5	C22H21D7O2	332.26014	6.39	0.13
Gestodene	GSD	310.43	C21H26O2	311.20056	5.41	0.18
Gestodene-d6	GSD-d6	316.47	C21H20D6O2	317.23822	5.37	0.19
Levonorgestrel	LNG	312.45	C21H28O2	313.21621	6.16	0.13
Levonorgestrel-d6	LNG-d6	318.49	C21H22D6O2	319.25387	6.11	0.14
Medroxyprogesterone acetate	MPA	386.52	C24H34O4	387.25299	7.45	0.16
Medroxyprogesterone acetate-d6	MPA-d6	392.56	C24H28D6O4	393.29065	7.40	0.11
Nomegestrol acetate	NOMAC	370.48	C23H30O4	371.22169	6.61	0.15
Nomegestrol acetate-d5	NOMAC-d5	375.51	C23H25D5O4	376.25307	6.59	0.13
Norethindrone	NOR	298.42	C20H26O2	299.20056	5.31	0.20
Norethindrone-d6	NOR-d6	304.46	C20H20D6O2	305.23822	5.27	0.18
Progesterone	P4	314.46	C21H30O2	315.23186	7.56	0.10
Progesterone-d9	P4-d9	323.52	C21H21D9O2	324.28835	7.47	0.12
Testosterone	T	288.42	C19H28O2	289.21621	5.71	0.15
Testosterone-d3	T-d3	291.44	C19H25D3O2	292.23504	5.69	0.15

traceptives was 1.24 [2] while the risk for ovarian, endometrial, and colorectal cancers [3] as well as osteoporosis is reduced [4]. However, in all these studies different progestins were considered together. Yet, depending on their chemical structure, individual progestins have different affinity towards the different hormone receptors and can act as agonists or antagonists to endogenous hormones [5,6]. To develop a more informed approach to hormonal contraception and combined hormone replacement therapy more differentiated analyses are required. With the advent of large-scale biobanking [7] and high-throughput analyses it has become feasible to examine large numbers of patient samples. In order to correlate plasma progestin levels with other clinical parameters, a high throughput approach to progestin measurements is required.

Historically, steroid hormone levels were determined by radio- or enzyme linked- immunoassays. These assays prevailed in the hospital/diagnostic setting due to their low upfront investment and ease of use. However, because they are based on antibody-antigen interaction, cross reactivity can be a problem, especially in complex matrices like plasma and urine, [8–10]. Moreover, for pediatric and mouse samples where sample volume is small, the number of hormones that can be simultaneously measured by these assays is limited. For instance, in case of murine blood sampling from the tail vein the final plasma volume one can obtain is usually <100 μl.

When gas chromatography coupled to mass spectrometry (GC–MS) was introduced, it soon became the method of choice for detection of steroids and helped characterize numerous aspects of steroid synthesis and metabolism [10]. However, due to the need of derivatization for GC–MS, it was increasingly surpassed by LC–MS [11]. With the evolution of LC–MS technologies and the expansion of their analytical applications, MS has become the technology of choice in bioanalysis despite the high upfront cost of the instrumentation and skill level requirement of the operator [10]. The current gold standard technical approach for LC–MS small molecule quantification is triple quadrupole technology (QqQ-MS) that provides high sensitivity and robustness through the selectivity of multiple reaction monitoring (MRM). Although high-resolution MS (HR-MS) with accurate mass determination capability is often applied for qualitative type analyses, it has recently been reported [12] that hybrid instruments like the QExactive (Thermo-Fisher, Waltham, MA, USA), can be an alternative to QqQ-MS technologies even for quantitative type analyses. Hybrid technologies combine the online filtering capacity of a quadrupole and the high resolution of the Orbitrap analyzer and allow both quantitative and qualitative analyses on a single instrument. Due to the lack of cumbersome reaction monitoring optimization, the HR-MS approach offers easier setup and technology transfer between laboratories. It can also be used to detect novel compounds in full scan (FS) mode which is not possible with QqQ-MS instruments due to their low resolution. Moreover, the need to simultaneously identify and quantify hundreds of metabolites drives a shift towards HR approaches [13–15].

Although endogenous steroid hormone panel analyses are commonly used in clinical analysis laboratories [16] and individual or small groups of synthetic progestin measurements by MS have been reported [17], the need for comprehensive profiling of progestin levels remains to be addressed.

Here, we have developed a high resolution LC–MS tSIM method to quantify a large panel of widely used progestins in small blood volumes for large scale studies of their effect on breast cancer risk and other diseases. To concomitantly assess the effect of these progestins on endogenous hormones we included a representative subset of endogenous steroids namely progesterone (P4), testosterone (T), androstenedione (A4), and cortisol (F) in the panel.

2. Materials and methods

2.1. Chemicals and reagents

Progesterone (P4), progesterone-d9 (P4-d9), testosterone (T), androstenedione (A4), cortisol (F), cortisol-d4 (F-d4), gestodene (GSD), gestodene-d6 (GSD-d6), levonorgestrel (LNG), levonorgestrel-d6 (LNG-d6), etonogestrel (ETO), etonogestrel-d7 (ETO-d7), chlormadinone acetate (CMA), chlormadinone

Table 2
Lower limits of quantification (LLOQ), %RSD, %Bias at LLOQ and calibration ranges.

Analyte	LLOQ (pg/ml)	%RSD	%Bias	Calibration range (ng/ml)
GSD	4.9	6.8	−11.2	0.16-100
LNG	19.5	1.7	−9.1	0.16-100
ETO	39.1	2.1	−8.5	0.16-100
CMA	78.1	2.0	−6.3	0.16-100
CPA	19.5	4.1	−0.7	0.16-50
DSP	2.4	4.6	−13.5	0.16-100
DAcNRG	39.1	1.8	−18.8	0.16-100
MPA	39.1	3.1	11.6	0.16-100
NOR	78.1	2.0	−8.6	0.16-100
DIE	19.5	1.5	−3.1	0.16-100
NOMAC	9.8	3.1	−7.0	0.16-100
P4	39.1	2.8	−15.9	0.16-100
T	39.1	1.9	−18.7	0.02-12
A4	39.1	4.4	−14.8	0.02-15
F	39.1	2.4	−18.7	0.82-500

acetate-d6 (CMA-d6), cyproterone acetate (CPA), cyproterone acetate-d3 (CPA-d3), drospirenone (DSP), drospirenone-d4 (DSP-d4), desacetyl norgestimate (DAcNRG), desacetyl norgestimate-d6 (DAcNRG-d6), medroxyprogesterone acetate (MPA), medroxyprogesterone acetate-d6 (MPA-d6), norethindrone (NOR), norethindrone-d6 (NOR-d6), dienogest (DIE), dienogest-d8 (DIE-d8) were all purchased from Toronto Research Chemicals (Ontario, Canada) testosterone-d3 (T-d3), androstenedione-2,3,4-^{13}C (A4-^{13}C), nomegestrol acetate (NOMAC) from Sigma, (St. Louis, MO, USA) and nomegestrol acetate-d5 (NOMAC-d5) were from Clearsynth (Mumbai, India). A list of the analytes and selected properties are shown in Table 1. Methanol, acetone (Thermo-Fisher, Waltham, MA, USA), water, acetonitrile (Biosolve, Dieuze, FR), 85% phosphoric acid (H_3PO_4) solution, acetic acid (Sigma, St. Louis, MO, USA) were all LC–MS grade. Double charcoal stripped human serum was from Golden West Diagnostics (Temecula, CA, USA).

2.2. Preparation of stock solutions, working standard solutions and calibration mixtures

Stock solutions (1 mg/ml) in methanol were stored at −80 °C. Working solutions in methanol contained: synthetic progestins and P4 (10 μg/ml), A4 (1.5 μg/ml), T (1.2 μg/ml), F (50 μg/ml). Deuterated or ^{13}C internal standard (IS) mix final concentrations in methanol were: for synthetic progestins and P4 (50 ng/ml), A4 and T (10 ng/ml), and F (125 ng/ml). Calibration and internal standard mixtures were stored at −80 °C. Nine point (including zero matrix point) calibration curves were established using double charcoal stripped human serum, with the following ranges: for synthetic progestins and P4 (0.16–100 ng/ml), for A4 (0.02–15 ng/ml), for T (0.02–12 ng/ml), and for F (0.82–500 ng/ml) (Table 2). Based on the necessity to measure concomitantly human and mouse samples as well as both endogenous and exogenous steroids we chose charcoal stripped human serum.

2.3. Biological samples

Human plasma samples were obtained from patients undergoing breast reduction surgery at the Centre Hospitalier Universitaire Vaudois (CHUV). The study was approved by the Commission cantonale d'éthique de la recherche sur l'être humain, ethics committee and informed consent was obtained from all patients. 8 to 12-week-old female NOD.Cg-*Prkdc*^{scid} *Il2rg* t^{m1Wjl}/SzJ (NSG) mice were used for hormonal pellet implantation. Mouse blood samples were collected in accordance with protocol VD1865.4 approved by the Service de la Consommation et des Affaires Vétérinaires of Canton de Vaud.

2.4. Hormonal treatment

Mice aged 10–20 weeks, were implanted subcutaneously with silicon based hormone pellets. After mixing part A, MP3745/E81949 and part B, MP3744/E81950 of low consistency silicon elastomer (MED-4011), hormone powder was added, mixed homogeneously, transferred to a syringe and incubated at 37 °C overnight. The polymerized mixture was removed from the syringe and cut into pieces containing the desired amount of hormone for a 60-day-release [18]. Blood was sampled 8 weeks later through tail vein or heart puncture.

2.5. Extraction

Solid phase extraction (SPE) on a MCX Oasis μElution 96-well plate was initially compared to liquid-liquid extraction (LLE) with Ethyl acetate: Hexane, (3:2). Although LLE recovery was superior, using a high throughput, 96 well format with the MCX plate far outweighed the benefit of slightly better extraction (Suppl. Fig. 1). 50 μl of human, mouse or calibrator plasma samples were mixed with 100 μl internal standard (IS) mix in 5% phosphoric acid solution and 50 μl H_2O for 10 min on orbital shaker. The acidified sample mix was applied to the SPE plate pre-activated with 200 μl methanol followed by 200 μl H_2O using a positive pressure manifold (Waters, Milford, MA, USA). After washing with 200 μl of 5% ammonium hydroxide (NH_4OH) and 200 μl of 20% methanol, steroids were eluted 2 x with 75 μl isopropyl alcohol (IPA) into a 96-deep well plate (Eppendorf, 500 μl). Eluates were evaporated under N2 on a TurboVap 96 (Biotage, Uppsala, Sweden) and reconstituted in 100 μl 50% methanol corresponding to initial LC mobile phase, plate was mixed 10 min using orbital shaker, centrifuged 5 min, 500 g and transferred to LC autosampler for analysis.

2.6. Liquid chromatography

The liquid chromatography was performed with a Dionex NCS -3500RS (Thermo-Fisher, Waltham, MA, USA) integrated with a ternary LPG micro pump and a heated column compartment. The Autosampler RSLCnano (Thermo-Fisher, Waltham, MA, USA) was configured for a 500 μL 96 well plate and a 125 μL loop. Briefly, 40 μL of sample were injected using μL pickup mode at 15 °C. Analytes were separated by reverse phase chromatography on a Zorbax Eclipse Plus C18 (2.1 × 50 mm 1.8 μm) column (Agilent Technologies, Santa Clara, California, United States), kept at 40 °C. Initial comparison was performed that determined MeOH/H_2O far superior to MeCN//H_2O for signal strength (Suppl. Fig.2). Further gains were achieved by using acetic acid in the mobile phase. The flow rate was 150 μl/min with the MeOH/H_2O mobile phase supplemented with 0.005% acetic acid Elution starting phase was set to 50% MeOH (0-0.1 min), linearly increased to 65% MeOH (0.1–2 min), further increased to 70% MeOH (2–2.01 min), held at 70% (2.01–5 min) then increased to 100% MeOH (5–5.01 min), maintained at 100% (5.01–7 min), decreased to 50% (7–7.5 min) and finally held at 50% MeOH for re-equilibration until the end of the 12 min run.

2.7. Mass spectrometry

Mass detection was carried out on a QExactive Orbitrap mass spectrometer in positive mode with the following parameters: resolution 70k, automatic gain control (AGC) target 3e^6, MAX ion injection time 50 ms, isolation window 1.5 m/z, scan range 250–450 m/z and heated electrospray ionization (HESI) source parameters: spray voltage 4 kV, capillary temperature 275 °C, nitrogen gas auxiliary temperature 330 °C, auxiliary gas flow rate 2, sheath gas flow rate 21, in source CID 5.0 eV. The tSIM method

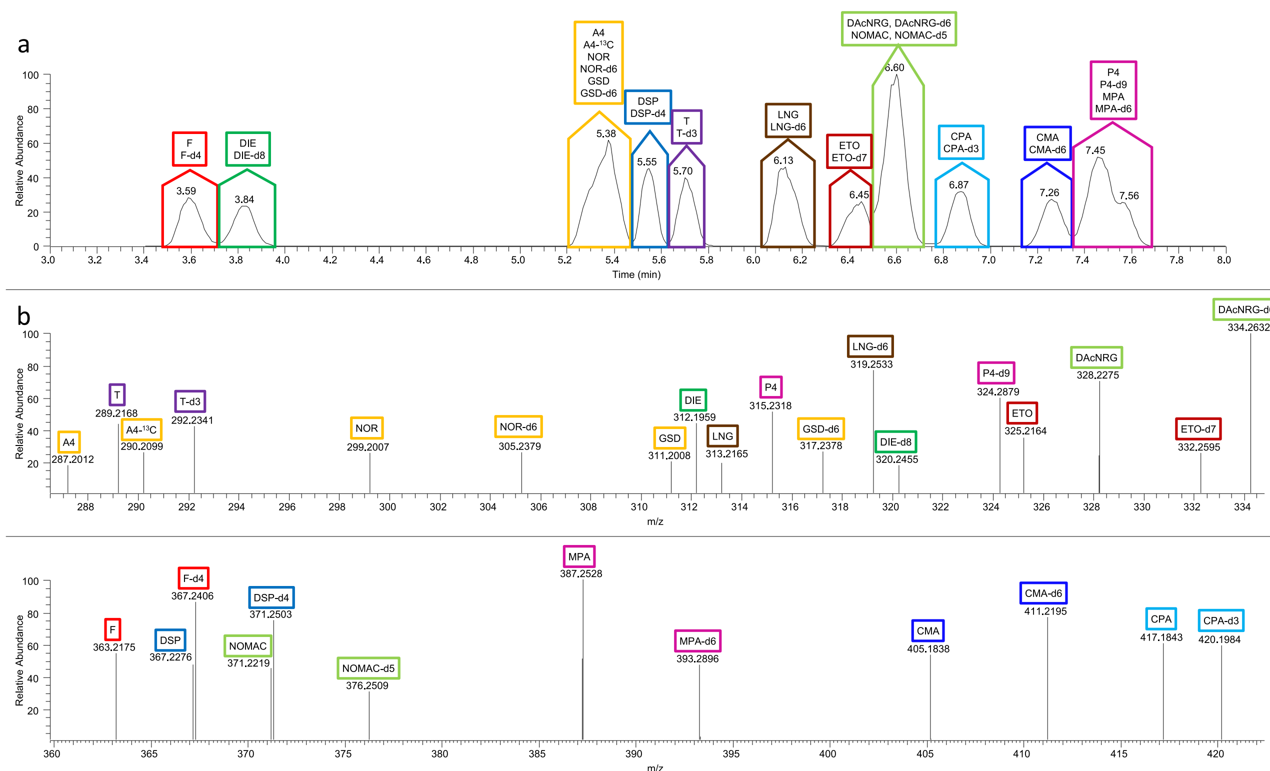

Fig. 1. Total ion current (a) and MS spectra (b) of all the analytes and internal standards detected in the tSIM method. Scan range was set between 250 and 450 m/z. Positive ion mode.

acquires the user defined targeted masses in t_R dependent time segments. In each segment the maximum number of target masses is set to 6, detected by msx count 6 multiplexing Fig. 1.

2.8. Validation

Method validation was performed according to FDA guidelines [19]. The following parameters were considered: intra and inter-day precision, selectivity, recovery, stability, calibration range linearity, carry over and retention time $(t)_R$ variation.

2.9. Data treatment

Acquired raw data was processed with Thermo Xcalibur 4.0.27.10. The targeted SIM method contained an inclusion list with 10 ppm (Suppl. Table 1) and exclusion list with 20 ppm precision. Mass extraction for analysis was carried out at 5 ppm.

3. Results

3.1. Selectivity, calibration curve and lower limit of quantitation (LLOQ)

Selectivity for calibrators and internal-standards was ensured through high resolution MS detection. Calibration ranges for endogenous compounds were selected based on clinically relevant concentrations [20]. For synthetic progestins, the ranges were selected according to expected human plasma concentrations [21]. Double charcoal-stripped human serum was used as matrix for the calibrator standards and QC samples. Calibration curves were constructed with 1/x weighted linear fitting within ranges shown in Table 2. Linearity values (r2) of the calibration curves attained 0.999 for all compounds but NOR 0.998, MPA 0.998 and CPA 0.996 (Suppl. Fig. 3). For CPA linearity was lost for concentrations > 50 ng/ml (Suppl. Fig.4). From the 8 non-zero calibrator levels only the lowest one for A4 and T do not comply with the criteria of having a < 10% RSD and < 20% bias required by FDA guidelines.

LLOQs were determined on quadruplicate samples serially diluted 1:2 over 15 dilutions. Quantification limit was set as < 10% RSD and < 20% bias, range linearity was maintained. LLOQs range from 2.4 pg/ml for DSP to 78.1 pg/ml for CMA and NOR. The majority of LLOQs were determined to be around 40 pg/ml with the exception of GSD, detected at 4.9 pg/ml, LNG and CPA at 19.5 pg/ml and NOMAC at 9.8 pg/ml (Table 2). Chromatograms of all target compounds at LLOQ are shown (Suppl. Fig.6).

3.2. Precision and stability

Repeatability (intra-day) and intermediate precision (inter-day) were determined by spiking 6 samples per low (L), medium (M) and high (H) concentration into charcoal stripped serum. For the synthetic progestins and P4 the concentrations were approximately 1, 10 and 100 ng/ml; for F they were 5, 50 and 500 ng/ml; for T 0.12, 1.2, 12 and for A4 0.15, 1.5 and 15 ng/ml. Intermediate precision was determined with 2 series of measurements performed on different days.

For most of the compounds, %RSD is < 10% for all tested levels with the exception of: DSP intra-, H (14.8%), and NOR intra-, H (10.8%), CPA inter- M (12.2%) and DSP inter- H (14.1%), with the majority of results being determined as < 5% (Table 3). For the high concentrations of CPA, there was an error that we attribute to the poor linearity of the calibration curve at the high end (Suppl. Fig. 3). This anomaly occurs because of interference between the 4th isotope of the non-deuterated calibrator standard and the first isotope of the deuterated internal standard. Isotopic distribution was calculated with ChemCalc [22]. The error becomes evident at high concentrations of the light standard like in the case of the highest calibrator (100 ng/ml) (Suppl. Fig. 3). Expected CPA levels in women on oral contraception are below 50 ng/ml (Table 3), yet in other applications such as treatment for hirsutism with high

Table 3
Validation parameters: Intra- and inter-day precision, post preparative stability, SPE recovery, matrix effect, carry-over.

		Intra-day		Inter-day		Post-prep. stability					
	Conc.	Added	Precision	Added	Precision	ST (%)		Recovery	Matrix	Carry-over	
Analyte	Level	(ng/ml)*	(%RSD)	(ng/ml)*	(%RSD)	24 h 15 °C	72 h −20 °C	SPE (%)	effect (%)	(‰)	(pg/ml)
GSD	L	1,2	3,0	1,2	3,2	99,7	99,7	85,8	11,6	0,33	0,39
	M	11,5	4,2	11,5	3,6	98,3	99,3	81,1	9,0	0,07	0,77
	H	106,0	3,9	105,1	3,7	105,2	98,3	83,1	6,0	0,07	*6,98*
LNG	L	1,2	1,9	1,2	2,0	101,6	100,2	84,6	9,0	0,18	0,22
	M	12,6	2,3	12,6	2,7	102,2	100,0	83,4	10,4	0,01	0,13
	H	100,2	6,2	100,2	5,2	105,2	100,0	83,9	7,1	0,11	10,76
ETO	L	1,3	3,6	1,2	3,7	101,0	96,6	82,0	6,8	0,16	0,21
	M	12,7	3,2	12,5	3,6	102,5	96,9	80,8	6,6	0,02	0,26
	H	101,7	3,9	101,1	3,6	106,5	98,8	82,8	5,4	0,08	7,82
CMA	L	1,3	2,8	1,3	4,8	100,1	108,1	76,6	23,7	6,65	8,32
	M	11,3	7,1	11,4	8,3	101,5	102,0	74,3	21,9	0,43	4,90
	H	100,2	4,3	99,1	3,6	103,5	99,0	73,4	14,4	0,09	9,33
CPA	L	1,5	3,0	1,7	8,7	100,6	99,4	81,7	33,2	0,00	0,00
	M	9,8	8,7	9,1	12,2	101,1	102,5	73,6	27,6	0,00	0,00
	H	193,9	8,0	190,9	7,0	97,5	74,4	75,9	11,7	0,00	0,00
DSP	L	1,2	2,7	1,2	2,7	99,2	105,6	79,0	9,8	0,09	0,10
	M	11,1	3,1	11,3	3,4	97,5	103,2	76,2	8,4	0,02	0,19
	H	120,2	14,8	111,9	14,1	98,0	102,0	77,9	6,5	0,06	*7,33*
DAcNRG	L	1,2	3,2	1,2	2,7	99,7	98,8	66,8	3,9	0,04	0,05
	M	11,9	2,4	11,9	2,5	99,9	99,4	66,6	3,2	0,02	0,27
	H	111,0	6,7	107,2	6,5	100,6	93,2	72,9	1,8	0,04	4,48
MPA	L	1,2	2,2	1,2	3,4	101,5	101,8	67,8	54,2	0,71	0,87
	M	11,3	2,8	11,2	3,1	102,1	102,1	74,9	17,7	0,05	0,55
	H	103,8	4,4	104,1	4,3	104,3	103,3	74,1	8,7	0,06	6,39
NOR	L	1,3	2,4	1,3	3,5	100,7	96,7	88,8	9,0	0,40	0,52
	M	12,7	2,8	12,8	2,9	99,9	100,4	86,8	10,4	0,03	0,35
	H	101,8	10,8	101,2	7,9	99,5	98,9	87,2	5,1	0,09	9,48
DIE	L	1,3	3,5	1,3	3,9	102,8	103,1	87,9	6,7	0,43	0,54
	M	12,8	5,9	12,7	5,5	105,9	99,5	83,8	0,7	0,01	0,11
	H	122,0	5,4	119,8	4,9	101,2	96,4	85,8	−5,5	0,05	5,62
NOMAC	L	1,1	3,7	1,1	3,3	99,7	99,0	79,3	9,7	0,05	0,05
	M	11,0	3,6	10,9	3,2	95,9	97,9	79,0	6,2	0,02	0,22
	H	104,5	3,8	103,6	3,9	96,7	98,4	83,2	2,9	0,05	4,85
P4	L	1,1	2,9	1,1	3,6	103,8	105,8	80,7	5,1	0,32	0,35
	M	11,4	2,4	11,4	2,3	102,4	100,4	79,6	6,2	0,03	0,39
	H	103,5	3,2	102,1	3,1	102,4	98,0	78,0	3,7	0,08	8,71
T	L	0,1	3,8	0,1	3,3	98,5	101,1	93,2	0,2	0,06	0,01
	M	1,4	3,0	1,4	2,6	96,0	99,8	85,5	5,6	1,96	2,73
	H	12,4	4,3	12,4	3,7	99,2	101,2	84,2	6,8	0,28	3,43
A4	L	0,2	7,8	0,2	9,1	97,7	102,7	89,6	−4,6	0,05	0,01
	M	1,7	4,6	1,7	4,8	97,3	97,7	81,8	8,7	4,01	6,77
	H	15,3	3,9	15,2	3,7	101,2	99,6	90,2	9,0	0,51	7,74
F	L	5,9	2,3	5,9	2,5	98,9	98,8	91,4	4,2	0,47	2,78
	M	57,6	1,8	57,7	2,1	98,7	100,0	90,7	4,1	0,04	2,15
	H	543,7	6,2	533,8	4,9	97,7	99,0	90,0	0,0	0,06	33,85

Italic: carry over > LLOQ.
* Avg. calc. added amt.

single dose, levels can exceed 200 ng/ml [23]. In this case, we recommend adapting the calibration range to suit expected analyte concentrations or the use of ^{13}C labelled internal standards with high enough mass shift compared to the non-labeled calibrator standard.

Steroid hormones are stable in plasma. Some steroids were reported to have degradation values during 4-day storage at room temperature within 2 standard deviations [24]. In our high throughput analytical set-up 24 h may pass between the first and last injection in the autosampler at 15 °C. Hence, post preparation stability may become important. To address this potential problem, we inserted an additional set of QC samples at the end of the analytical series.

As instrument access and scheduling can be an issue during measurements we also tested whether a prepared sample plate could be stored at −20 °C for over 72 h before injecting. All the compounds had stability values > 93% indicating that storage of the processed samples for at least 72 h does not impact significantly the final measurements (Table 4).

3.3. Recovery, matrix effect and carry over

SPE on the MCX micro elution plate was optimized for the recovery of the low abundant endogenous analytes A4 and T. SPE recovery was determined by comparing pre- and post-extraction spiked charcoal stripped serum, 4 replicates each, spiked to low, medium and high (L, M, H) concentration levels described above. The recovery range was between 66.6% for DAcNRG and 95.1% for A4 (Table 3). The repressive effect of the matrix on ionization of the compounds was determined on quadruplicate samples with and without extracted human blank serum. For 13 out of 15 compounds the extraction recovery was > 75%. The exceptions were MPA with 67.8% for the L concentration sample and DAcNRG with 66.8%, 66.6% and 72.9% for the L, M and H concentration sample, respectively. Matrix effect was < 20% for all compounds but MPA which fared poorly with 54.2% in case of the L concentration sample (Table 4).

Carry-over of analytes due to sample preparation, injection system, or column retention was assessed by injecting a blank,

Table 4
Method validation for human and mouse samples. Table showing treatment, progestin identity, measured concentration, reported Cmax and half-life.

Species	Treatment	Progestin	Conc. (ng/ml)	*C_{max} (ng/ml)	$t_{1/2}$ (h)
Human	OC	GSD	3,84	3,6	12-14
	OC	GSD	3,79	3,6	12-14
	OC	GSD	3,54	3.6	12-14
	OC	LNG	3,63	2.5-6	9.9-13.2
	OC	LNG	1,53	2.5-6	9.9-13.2
	OC	LNG	1,42	2.5-6	9.9-13.2
	OC	DSP	35,36	32.1-41.7	31.1-32.5
	OC	DSP	31,05	32.1-41.7	31.1-32.5
	OC	DSP	28,02	32.1-41.7	31.1-32.5
	OC	ETO	4,13	2.72-4.66	11.9-23.8
	OC	ETO	2,07	2.72-4.66	11.9-23.8
	OC	ETO	2,82	2.72-4.66	11.9-23.8
	OC	CPA	6,67	8.6-21.8	54-78.6
	OC	CPA	8,48	8.6-21.8	54-78.6
	OC	CPA	5,84	8.6-21.8	54-78.6
	IC	MPA	2,85	1-7	24
	OC	MPA	1,78	1-7	24
	OC	MPA	1,79	1-7	24
Mouse	HP	GSD	2,84		
	HP	GSD	3,63		
	HP	GSD	2,66		
	HP	LNG	2,1		
	HP	LNG	2,9		
	HP	LNG	6,02		
	HP	DSP	23,56		
	HP	DSP	35,57		
	HP	DSP	29,31		
	HP	ETO	1,71		
	HP	ETO	4,28		
	HP	ETO	4,25		
	HP	CPA	4,34		
	HP	CPA	3,52		
	HP	CPA	1,54		
	HP	MPA	6,12		
	HP	MPA	9,78		
	HP	MPA	6,97		
	HP	CMA	1,24		
	HP	CMA	1,75		
	HP	CMA	1,54		

OC - oral contraceptive.
IC - injected contraceptive.
HP - hormone pellet.
* reported in human plasma.

Table 6
Endogenous hormone measurements from treated mouse samples.

Treatment	P4 ng/mL	A4 ng/mL	T ng/mL
Control	17.86	0.041	0.051
Control	1.272	0.109	0.167
Control	6.615	0.14	0.19
Control	5.423	0.104	0.158
P4 pellet	42.806	0.058	0.309
P4 pellet	41.537	0.136	0.196
P4 pellet	16.65	0.19	0.132
P4 pellet	44.775	0.116	0.097
Pregnant	59.619	<0.04	0.432
Ovariectomized	3.321	0.08	0.24

non-extracted sample consisting of initial mobile phase conditions after the spiked plasma samples (Table 4). Carry-over was calculated as the per-mille peak area detected in the blank after a previously injected spiked sample. From both the spiked sample and following blank peak areas the non-carryover background peak area was subtracted. We noticed a low analyte carryover throughout the sample series for all species measured on the analytical setup described here thereby alleviating the need for intercalated blanks with the benefit of significantly decreasing the total time of analysis. Nonetheless, for GSD and DSP the carryover values after injection of highly concentrated samples were higher than their respective LLOQs. However, they were still much smaller than the typical analyte concentrations found in primarily human and also mouse plasma samples that the method is intended for (Tables 5 and 6,). These carry-over amounts are represented in italic in Table 4.

3.4. Method application: Determination of progestins in human and mouse plasma

Next we validated the method in biologically accurate matrices like mouse and human plasma. This step is important from the point of view of matrix effects including interference and specificity in a complex background. In order to validate the method for human samples, we measured progestin levels in the plasma of women who declared to be on contraceptives. Two different patient samples were measured for each progestin taken in the context of oral contraception at the time of blood collection. In all cases, the progestin in question was detected by the method with the following results in comparison to the reported peak concentration (Cmax): GSD: 3.84 and 3.79 with a reported Cmax of 3.6 ng/ml; LNG: 3.63 and 1.53 whereas the Cmax ranges between 2.5–6 ng/ml; DSP: 35.36 and 31.05, Cmax 32.1–41.7 ng/ml; ETO: 4.13 and 2.07, Cmax 2.72–4.66 ng/ml; CPA: 6.67 and 8.48, Cmax 8.6–21.9 ng/ml; MPA: 2.85 and 1.78, Cmax 1–7 ng/ml. Even though the human plasma concentrations of the different progestins vary depending on the dose administered and the time elapsed between last intake and blood withdrawal, the method can positively identify the progestogen component of the oral contraceptives taken with a value that is close to the reported Cmax value for the particular progestin (Table 3) [21].

As biological samples containing DAcNRG, NOR, DIE and NOMAC, were not available, we checked and validated the method

Table 5
Endogenous hormone measurements and their reference intervals, from pre-and post-menopausal women samples.

Menopause	Age	P4 (ranges)		A4 (ranges)		T (ranges)		F (ranges)	
	Years	ng/mL		ng/mL		ng/mL		ng/mL	
NO	18	0.081	(<17.816)	1.653	(0.277-1.638)	0.498	(0.104-0.454)	140.368	(47.4-199.7)
NO	19	1.054	(<17.186)	1.023	(0.277-1.638)	0.29	(0.104-0.454)	43.924	(47.4-199.7)
NO	23	0.05	(<17.816)	0.556	(0.277-1.638)	0.213	(0.104-0.454)	43.221	(47.4-199.7)
NO	33	0.726	(<17.816)	1.307	(0.277-1.638)	0.428	(0.104-0.454)	68.051	(47.4-199.7)
NO	40	4.862	(<17.816)	1.351	(0.277-1.638)	0.331	(0.104-0.454)	23.818	(47.4-199.7)
NO	41	13.983	(<17.816)	0.337	(0.277-1.638)	0.167	(0.104-0.454)	27.131	(47.4-199.7)
YES	68	0.047	(<0.08)	0.569	(0.095-0.773)	0.268	(0.077-0.392)	102.788	(56.9-180.4)
YES	70	0.033	(<0.08)	0.34	(0.095-0.773)	0.373	(0.077-0.392)	86.648	(56.9-180.4)
YES	71	0.024	(<0.08)	0.413	(0.095-0.773)	0.236	(0.077-0.392)	38.658	(56.9-180.4)
YES	73	<0.04	(<0.08)	0.352	(0.095-0.773)	0.233	(0.077-0.392)	92.109	(56.9-180.4)
YES	74	0.043	(<0.08)	0.932	(0.095-0.773)	0.2	(0.077-0.392)	110.441	(56.9-180.4)
YES	75	0.047	(<0.08)	0.661	(0.095-0.773)	0.445	(0.077-0.392)	176.865	(56.9-180.4)

for these 4 progestins by spiking them into human matrix and checking for matrix interference and performing all the validation steps, as described above.

To determine steroid levels in murine plasma, we collected blood from mice implanted subcutaneously with silicon-based pellets containing different progestins. Measurements on plasma from mice implanted with progestin pellets were as follows: GSD: 2.84, 3.63 ng/ml; LNG: 2.1, 2.9 ng/ml; DSP: 23.56, 35.57 ng/ml; ETO: 1.71, 4.28 ng/ml; CPA: 4.34, 3.52 ng/ml; MPA: 6.12, 9.78 ng/ml; CMA: 1.24, 1.75 ng/ml (Table 3).

In the murine samples, concentrations varied depending on the time of pellet implantation, with a peak in the first couple of days that tapers off towards the end of the pellet lifetime which was about 60 days as reported in pharmacological studies with pellet implants [25]. Pellet sizes were determined to recapitulate the progestin levels found in the blood of women on contraceptives.

In order to prove the validity of the method for endogenous hormones as well, we compared A4, P4, T and F measurements from both pre-and post-menopausal women with previously published reference intervals [26] (Table 5). Additionally endogenous A4, P4 and T (F not being relevant for mice, [27]) levels were measured from control, ovariectomized, pregnant and P4 pellet treated NSG mouse plasma (Table 6). Representative chromatograms of endogenous hormones measured in real samples are shown in Suppl. Fig.7.

4. Discussion

Here we provide a robust HR-MS method for detecting and quantifying a comprehensive progestin panel in clinical samples, which is easy to implement. The method proved to be highly reproducible, selective and precise for 11 progestins and 4 endogenous steroid hormones. Compared to values obtained with GC–MS/MS approaches that require additional derivatization [28] or other LC–MS/MS [11] approaches, these values are in the same range. However, one needs to consider the starting material quantity as well as matrix complexity when comparing LLOQs. Consequently, the sensitivity of the method is sufficient for quantification of these compounds in clinically relevant concentration ranges and comparable to that of other published methods.

The advantage of a scheduled targeted SIM method on a QExactive instrument in regard to selectivity and sensitivity lies in the pre-filtering of ions by the quadrupole and high mass accuracy and resolution of the Orbitrap. The reduced ion population post-quadrupole lowers the risk of any post-interface ion suppression that may occur in the C-trap and the pre-filtering of the quadrupole improves the signal-to-noise ratio (S/N), by increasing the number of targets ions scanned in the Orbitrap, thereby lowering the detection/quantification limits. Additionally, in order to increase selectivity in a complex background like plasma, the instrument was used in a high resolution mode (70k) typically associated with undesirable longer transient times (250 ms) and longer cycle times. For example, for a chromatographic peak of 15 s in order to have at least 10 scans per peak, cycle time needs to be 1.5 s or shorter. In this method, cycle times were reduced by using a short scan range (250–450 m/z), careful analyte scheduling, and multiplexing of the tSIM scans for quantitation of co-eluting targets. Accurate scheduling requires highly reproducible compound retention times reflected by < 0.2 coefficient of variation (CV) for 25 out of 30 analytes (Table 1). The method was also validated on human and mouse plasma samples, in each case, the progestins could be positively identified and quantified. Nevertheless, there are several limitations. Firstly, by stripping the serum to remove steroids we may simultaneously remove potential interferences, which in such a case would not be present during method validation. Furthermore, the presence of matrix effects is unnoticeable during the validation process and it should be considered that there could potentially be a decrease in the precision of the method due to this effect. On the other hand, the representative chromatograms of real samples (Suppl. Fig.7) show that the tSIM method is inherently less sensitive to matrix effects due to the high pre-filtering of the quadrupole, which results in high signal to noise S/N ratios.

In the context of clinical studies our method can help build databases that contain exact hormone levels and do not rely on the commonly inaccurate questionnaire information provided by study participants. If widely implemented, the described method can become the source of a reliable, large dataset that can open unprecedented opportunities into evidence-based cohort studies focusing on the effects of different progestins on various diseases.

As we strive toward precision medicine frequent and reliable hormone measurements should be standard procedure both in the clinic as well as in research laboratories. By enabling frequent monitoring of plasma steroid levels, our method can help build breast cancer models that accurately mimic human steroid/progestin blood levels which are highly sought after attributes of pre-clinical models.

CRediT author statement

CFL: project administration, methodology, investigation, formal analysis, data curation, validation, writing- original draft - review and editing; JPM: methodology, writing- review and editing; MS: resources; FDM: data processing, review, editing; AB and RN: resources; SJB and MM: resources, supervision, writing- review and editing, HH: conceptualization, methodology, resources, writing-review and editing; CB: conceptualization, writing- original draft - review and editing, supervision, funding acquisition.

Declaration of competing interest

None.

CRediT authorship contribution statement

Csaba Ferenc Laszlo: Project administration, Methodology, Investigation, Formal analysis, Data curation, Validation, Writing - original draft, Writing - review & editing. **Jonathan Paz Montoya:** Methodology, Writing - review & editing. **Marie Shamseddin:** Resources. **Fabio De Martino:** Writing - review & editing. **Alexandre Beguin:** Resources. **Rene Nellen:** Resources. **Stephen James Bruce:** Resources, Supervision, Writing - review & editing. **Marc Moniatte:** Resources, Supervision, Writing - review & editing. **Hugues Henry:** Conceptualization, Methodology, Resources, Writing - review & editing. **Cathrin Brisken:** Conceptualization, Writing - original draft, Writing - review & editing, Supervision, Funding acquisition.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jpba.2019.07.004.

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