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RESEARCH ARTICLE

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Rapid UHPLC–MS/MS measurement of pregnanediol 3-glucuronide in spot urine samples for detecting ovulation

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Abstract

Biochemical confirmation of ovulation typically involves measuring serum progesterone levels during the mid-luteal phase. Alternatively, this information could be obtained by monitoring urinary excretion of conjugated metabolites of ovarian steroids such as pregnanediol 3-glucuronide (PDG) using immunoassay techniques that have methodological limitations. The aim of the present study was to develop a mass spectrometry (MS)-based method for the rapid and accurate measurement of urinary PDG levels in spot urine samples. A "dilute and shoot" ultra-high-performance liquid cromatography tandem mass spectrometry (UHPLC-MS/MS) method was developed for measuring PDG urinary concentration with a 6-min analysis time. The method underwent validation in accordance with ISO 17025 documentation for quantitative methods, proving an efficient separation of PDG from other structurally similar glucuro-conjugated steroid metabolites and ensuring a sufficient sensitivity for detecting the target analyte at concentrations as low as 0.01 µg/mL. The validation protocol yielded satisfactory results in terms of accuracy, repeatability, intermediate precision, and combined uncertainty. Additionally, the stability of both the samples and calibration curves was also conducted. The application to real urine samples confirmed the method's capability to measure PDG levels throughout an entire menstrual cycle and detecting ovulation. The rapidity of the analytical platform would therefore enable high throughput analysis, which is advantageous for large cohort clinical studies.

KEYWORDS
dilute and shoot, LC–MS/MS, ovulation, pregnanediol glucuronide, urine

1 | INTRODUCTION

There is considerable interest in the detection of ovulation due to its pivotal role in the reproductive cycle. The World Health Organization (WHO) conducted a large multinational study to determine sex distribution and infertility etiologies. Within the same study, anovulation and ovulatory disorders were identified as primary factors contributing to female infertility, accounting for 20% to 25% of causes of

reproductive failure (WHO, 1992). The ability to predict or detect ovulation is equally essential for procedures such as artificial insemination or natural family planning.

Practical techniques for ovulation detection and timing may be classified into two groups: (1) evaluation of peripheral changes preceding, coinciding with, or succeeding the ovulatory process, and (2) direct assays of gonadotropins or steroid hormones in the serum, urine, or saliva.

In cases of menstrual cycle irregularities or for women planning conception or undergoing infertility investigations or treatment, biochemical confirmation of ovulation is necessary.

Biochemical tests are generally based on hormonal assays and include the following:

1. Home ovulation predictor kits, which measure urine LH levels to detect the LH surge preceding ovulation.
2. Mid-luteal progesterone testing or urinary excretion of conjugated metabolites of ovarian steroids (pregnanediol 3-glucuronide [PDG] and/or estrone 3-glucuronide [EG]) measured by immunoassay. These tests facilitate the retrospective determination of ovulation occurrence by detecting progesterone produced by the corpus luteum.

More specifically, progesterone (P4) plays a central role in the menstrual cycle. During the luteal phase, P4 prepares the endometrium of a woman's uterus to receive and nourish a fertilized egg, and guarantee the implantation process (Goletiani et al., 2007). P4 is not expected to be metabolized or excreted in the same way by each woman (Ellison et al., 1993; Lawrence et al., 2018). Despite this, elevated serum P4 concentrations are therefore specific for the post-ovulatory luteal phase of the menstrual cycle in non-pregnant humans, leading to its wide use to confirm recent ovulation. However, the use of serum samples requires venipuncture by a healthcare professional during the mid-luteal phase, limiting its practicality for self-testing at home.

Estimating P4 levels using its major metabolite, PDG, in urine could be an alternative approach to avoid interference caused by individual hormone differences (Mithileshwari et al., 2016) and the invasiveness of sampling. This is feasible due to the interchangeability of P4 and PDG as ovulation markers, as they both peak around 7–8 days after ovulation (Roos et al., 2015).

Over the years, numerous immunochemical tests have been developed to measure PDG in urine (Collins et al., 1979; Stanczyk et al., 1980). Initially, these tests required extensive 24-h urine sampling, but they were later simplified to allow for spot urine sampling (Metcalf, 1976), including adaptations for measurement using dried pH strips (Wasalathanthri et al., 2003).

Enzyme immunoassay (EIA) is a widely used technique for measuring PDG, functioning on the principle of antigen–antibody binding (Hama et al., 2009). This method is advantageous due to its low cost, ease of use, and rapid results (Kulle et al., 2017). However, EIA measures tracers rather than directly detecting analytes, potentially causing specificity issues due to antibody cross-reactivity and other interferences from the sample matrix (Ismail et al., 2002). Furthermore, the lack of standardization in immunoassays complicates comparison with laboratory results, often necessitating consideration of specific reference ranges (Kulle et al., 2017).

In contrast, steroid determination via mass spectrometry (MS) is a physico-chemical approach that identifies molecules by measuring the m/z of precursor or product ions. Regarding gas chromatography–mass spectrometry (GC–MS), there have been available methods to measure non-conjugated pregnanediol (PD) since the 1960s (Cox, 1963; Barrett & Brown, 1970). These methods are used in doping control analyses to monitor PD as an endogenous reference compound (Brooker et al., 2012). However, in GC–MS analysis, conventional sample preparation procedures involve an enzymatic hydrolysis step to release the aglycones, resulting in the loss of information on the free or sulfo-conjugated fraction. Following this, samples are preconcentrated and analyzed by GC–MS after derivatization (Badoud et al., 2013; Van Renterghem et al., 2010; Strahm et al., 2008). These steps can be time-consuming and can introduce variations, technical errors, and inaccuracies (Mareck et al., 2008).

Compared to traditional isotopic and non-isotopic EIAs and GC–MS/MS, LC–MS/MS offers greater specificity (Cox, 1963; Otero-Fernàndez et al., 2013). Due to its superior sensitivity and selectivity, LC–MS/MS is already extensively used in the quantitative analysis of hormones within the public health field (Michely et al., 2017).

Moreover, LC–MS has already proven to be also the ideal technique to analyze phase II metabolites of EAAS without the need for hydrolysis (Esquivel et al., 2018, 2019; Pozo et al., 2008). Some research groups have analyzed intact glucuronides (Pozo et al., 2008), intact sulfates (Esquivel et al., 2019), or a combination of both (Badoud et al., 2011; Boccard et al., 2011; Schulze et al., 2011).

Concerning ovarian steroids, the initial liquid chromatography-mass spectrometry (LC–MS) method to measure urinary excretion of conjugated metabolites of sexual steroids (Bowers & Sanaullah., 1996) did not include PDG, while the first MS-based technique for measuring PDG in urine employed an outdated hard ionization method (Moneti et al., 1985). More recent LC–MS methods have been reported to measure PDG in serum using methanol as extraction solvent (Chen et al., 2020).

A recent study has demonstrated promising results for the measurement of PDG concentrations in dried urine spots (DUS) by LC–MS without the need for deconjugation or derivatization, using methanol-diluted urine (Handelsman et al., 2021). All these previously cited methods apply solid-phase extraction (SPE) as sample preparation step or involve an extended extraction process prior to the LC–MS/MS analysis. However, it has been demonstrated in various studies that even by using a "dilute and shoot" LC–MS/MS approach, steroid phase II metabolites can also be detected (Deventer et al., 2014; Pozo et al., 2008).

The aim of the present work is to develop a "dilute and shoot" ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) method for the rapid and accurate measurement of PDG urinary concentration, using a minimal volume of spot urine sample.

In this method, the urine matrix is analyzed by UHPLC–MS without undergoing pre-analytical treatment. Typically, non-extracted urine is a matrix that compromises column performance and lifespan (De Wilde et al., 2020). Therefore, this work aims to address the issue of columns degradation and to reduce analysis time by implementing a pre-analytical procedure that involves only two steps before instrumental MS-analysis.

2 | MATERIALS AND METHODS

2.1 | Chemical and reagents

Certified reference material for pregnanediol 3-glucuronide (PDG) and the labelled internal standard (PDG ^{13}C5, IS) for the target steroidal compound were obtained from Merck KGaA (Darmstad, Germany). UHPLC/MS grade methanol (MeOH) was purchased from Carlo Erba Reagents (Cornaredo, Italy); ammonium fluoride (NH_4F) for MS was supplied by Merck KGaA (Darmstadt, Germany). Ultra-pure water (18.2 MΩ × cm) was obtained using the Smart2pure® system (Thermo Scientific, Waltham, MA, USA) and was used for the preparation of all LC mobile phases and aqueous solutions. Synthetic urine (Sigmatrix Urine Diluent) was also acquired from Merck KGaA (Darmstadt, Germany). Stock solutions of the analyte and IS was prepared at a concentration of 1 mg/mL in MeOH and stored in 1.5 mL amber glass vials at −80°C. Working solutions at appropriate concentrations were prepared by serial dilution of the stock solution in MeOH and stored in 2 mL amber glass vials at −80°C. The dilution solution, containing IS at 100 ng/mL, was prepared spiking 1 mL of PDG ^{13}C5 at 10 µg/mL in 100 mL of a MeOH/H_2O solution (50/50, v/v), which was stored in a 150-mL bottle at −20°C and used for the sample preparation procedure.

2.2 | Calibration and validation samples

For the preparation of calibration and validation samples, working solutions containing PDG were prepared through sequential dilution of the stock solution of PDG at different concentration levels in MeOH (100, 10, 1 µg/mL). Calibration and validation samples were then prepared by spiking the synthetic urine with these working solutions containing PDG to achieve the desired final concentrations (details presented in Supplementary Material Table S1).

2.3 | Samples preparation and UHPLC–MS/MS analysis

Urine samples and calibrators (7 µL each) were pipetted into 1 mL 96-well plates, followed by the addition of 693 µL of cold MeOH/H_2O (50/50, v/v) fortified with the isotope labelled IS (PDG ^{13}C5). The dilution solution (700 µL) was used as a blank sample. The mixture was then shaken for 10 min at 350 rpm. Subsequently, 20 µL of each sample, calibrator and blank were finally injected into the UHPLC–MS/MS system for analysis.

Analyses were carried out using a Nexera X2 UHPLC system (Shimadzu, Tokyo, Japan) coupled to a Citrine Triple Quad MS/MS system (AB Sciex, Ontario, Canada). The *Analyst* software was used to control the system, while the *MultiQuant* software (AB Sciex, Ontario, Canada) was used to perform data analysis (peak integration and quantification). Chromatographic separation was carried out using a Luna® Omega C18 analytical column (100 × 2.1 mm, 1.6 µm; Phenomenex, Torrance, USA) set at 50°C. Mobile phase A was 2 mM NH_4F in H_2O, and mobile phase B was MeOH. Chromatographic gradient started with a 0.5-min isocratic step at 50% B, followed by an increase from 50% to 98% in 3.5 followed by a washing step at 98% B for 1 min; the column was then re-equilibrated at the initial conditions for 1 min. The injected volume was 20 µL, and the flow rate was set at 400 µL/min, with a total run time of 6 min.

MS/MS analysis was performed in negative ionization mode using an electrospray ionization (ESI) source with the following parameters: source temperature was set at 50°C, the ion spray voltage was set at −4500 V, and the pressures for the curtain gas, nebulizer gas, and heater gas pressures were set at 35, 40, and 50 psi, respectively. Details of the optimized MS/MS parameters for PDG and PDG ^{13}C5 are reported in Table 1.

2.4 | Method validation procedure

The UHPLC–MS/MS method was validated using a free online software called "Methods Validation App" (MVA) (Solarino et al., 2024), developed by the Department of Chemistry at the University of Turin. The logic and operation of this software are explained in a previously published work (Alladio et al., 2020).

This operation follows the preparation and analysis of three independent replicates for each calibration level in three different analytical sessions performed in three different days. Through this, it is

TABLE 1 Optimized mass spectrometric parameters for target analyte and internal standard MRM transitions.

Analyte	Ionization mode	Q1 mass (Da)	Q3 mass. (Da)	DP (V)	EP (V)	CE (V)	CXP (V)
Pregnanediol glucuronide (quantifier)	Neg	495.2	113.1	−190	−10	−42	−13
Pregnanediol glucuronide (qualifier)	Neg	495.2	74.8	−190	−10	−92	−11
Pregnanediol glucuronide ^{13}C5 (IS)	Neg	500.2	74.8	−185	−10	−92	−13

Abbreviations: CE, collision energy; CXP, cell exit potential; DP, declustering potential; EP, entrance potential; MRM, multiple reaction monitoring.

possible to evaluate the potential heteroscedasticity of the instrumental signal and therefore perform weighted regression with first- or second-degree polynomials. Additionally, it is possible to automatically calculate other parameters such as limit of detection (LOD), inter-day and intra-day precision, and accuracy.

In order to be compliant with the request of ISO/IEC 17025:2017 documentation (ISO. International Organization for Standardization, 2017), we also included in the validation protocol the assessment of matrix effect, selectivity, repeatability, carry-over, and robustness. Furthermore, the stability of both prepared samples and calibration curves stored at 4°C for up to 1 week was evaluated.

2.4.1 | Calibration

To calculate the appropriate calibration curve for each analyte, the uniformity of variance within the calibration range was initially analyzed. This was done using Levene's test to compare the variance associated with the nine replicates for each calibration level. The Brown–Forsythe version of the test was used to ensure robustness even in the case of non-parametric distributions.

If the system is heteroscedastic, a regression will be performed without and with a weighting factor of $1/x$ if the variance of the signal increases linearly with concentration, or using a weighting factor of $1/x^2$ if the variance increases quadratically. The chosen weighting factor will be the one that generates the least variance following the interpolation process. Finally, the degree of the polynomial is decided by conducting a Mandel test, comparing the sum of squares of the regression of the linear model and the quadratic model using an *F*-test.

2.4.2 | Accuracy

Accuracy, defined as the closeness of the experimental data to the true value, was calculated in terms of intra-day and inter-day bias%. The bias% is calculated using the following equation:

$$\left[\text{bias}\% = \left(1 - \frac{x_{\text{exp}}}{x_{\text{real}}}\right) \times 100\right]$$

where x_{real} is the known concentration value for a given calibration level and x_{exp} is the mean of the measured concentration values for the same level. The calculation of intra-day accuracy is performed by using two out of three replicates conducted each day to calculate a calibration curve with weights and polynomial degree in accordance with the previous step. The instrumental signals of the third replicate are then interpolated, and the mean of the resulting concentrations is calculated.

The calculation of inter-day accuracy follows a similar scheme: concentration values for a given day are calculated by interpolation with a calibration curve constructed using the instrumental signals from the remaining two days; this is done for each day, and the results for each calibration level are averaged and used to calculate the bias %. Accuracy values obtained were considered optimal if the calculated bias% was less than 15% and acceptable if it was between 15% and 20%.

2.4.3 | Precision

Precision, understood as the reproducibility or agreement between measurements of the same quantity, was calculated as the coefficient of variation percentage (CV%) intra-day and inter-day according to the following equation:

$$\left[\text{CV}\% = \frac{\sqrt{\sum_{j=1}^{J}\left(x_{\text{exp},j} - \bar{x}\right)^2/(J-1)}}{\bar{x}} \times 100\right]$$

where J is the number of replicates, x_{exp} is the result of the jth replicate, and $\bar{x}$ is the mean of all replicates for a given calibration level.

For the calculation of intra-day and inter-day precision, the MVA software uses the same backward calculation protocol as seen for accuracy: the instrumental signals for each level of a calibration curve are used and interpolated with the curve calculated from the remaining data. For validation purposes, precision values were considered optimal if below 15% and acceptable if between 15% and 20%.

2.4.4 | LOD and LLOQ

The LOD was calculated with the Hubaux-Vos algorithm. This algorithm allows for the calculation of the minimum detectable concentration using the calibration curve data and the error associated with the regression, defining two confidence intervals from which it is possible to estimate with a certain degree of significance the instrumental responses that would generate false positive and false negative results. This method for calculating the LOD was initially developed for homoscedastic data distributions, but it can also be used for heteroscedastic distributions by introducing weighting factors. The lower limit of quantification (LLOQ) of each analyte was defined as the lowest tested concentration level for which intra- and inter-day precision, and accuracy values lower than 20% were measured.

2.4.5 | Matrix effect

The evaluation of the matrix effect was conducted by analyzing, in triplicate, a real urine sample with a measured PDG concentration below LLOQ fortified with PDG to achieve a final concentration of 50 μg/mL, alongside a methanolic solution of 50 μg/mL PDG. Furthermore, the three fortified real urine samples were also compared with three fortified synthetic urine samples each with a PDG concentration of 50 μg/mL, to assess the suitability of the synthetic matrix for preparing calibration and validation samples.

2.4.6 | Selectivity

The selectivity assessment involved preparing and analyzing four Level 5 calibration samples, four real female urine samples, and four real male urine samples. Fragment ion ratios measured in calibration samples were compared with those observed in real urine samples. Additionally, 24 different synthetic urine samples were created by spiking each with a methanolic solution containing one single steroid glucuronide at a concentration of 10 ng/mL (the list of all investigated steroid glucuronides is provided in Supplementary Material Table S2). These samples underwent the sample preparation procedure and were analyzed to investigate potential chromatographic interferences affecting the selected MS/MS transitions of target analyte in its elution region.

2.4.7 | Carry-over

The carry-over effect was assessed by analyzing three synthetic urine samples immediately after the most concentrated calibration sample (Level 8). Carry-over was considered negligible if the mean peak area of the target analyte in synthetic urine samples was less than 1% of the peak area measured in the Level 8 calibration sample.

2.4.8 | Robustness

During the three days of the quantitative validation protocol, the impact of minor changes including operator sampling extraction, mobile phase preparation, analytical column lot, and multiple instrumental maintenance was evaluated to assess the method's robustness.

2.4.9 | Stability

The stability of prepared samples was assessed by analyzing quality control (QC) urine samples (low, medium, high) in triplicate on the day of their preparation and again after 4 and 7 days of storage at 4°C. The same approach was used to evaluate the stability of prepared calibration curves. Calibration curves were initially prepared and analyzed during the first series of validation and then stored for 4 and 7 days at 4°C before being used to quantify freshly prepared QC urine samples.

2.5 | Real sample application

To evaluate the efficiency of the validated UHPLC–MS/MS method, two different sets of real urine samples were analyzed. The first set included urine samples collected from seven healthy females (mean age 27.1 years, mean BMI 19.7 kg/m^2), recruited at the Division of Endocrinology, Diabetology and Metabolism of the Verona University Hospital. For each volunteer, four urine samples were collected throughout the duration of a menstrual cycle (except for one individual from whom only three samples were collected), specifically at 7, 14, 21, and 24 days after the cycle's start, totaling 24 samples. The second set comprised 24 samples collected from a single healthy female (age 34 years, BMI 22.0 kg/m^2) recruited at the City of Health and Science Turin University Hospital. One sample per day of the menstrual cycle was collected until the reappearance of menstrual flow, establishing a longitudinal profile for a single person. All enrolled individuals provided consent for the use of urine samples for research in accordance with the Declaration of Helsinki. Once collected, samples were immediately anonymized and stored at −80°C until analysis. In addition to UHPLC–MS/MS quantitative analysis of PDG, urinary creatinine was measured using an enzymatic test performed with the AU680 Clinical Chemistry Analyzer (Beckman Coulter, Brea, CA, USA) to normalize PDG concentrations measured in the collected spot urine samples.

3 | RESULTS AND DISCUSSION

3.1 | Method development

In the preliminary stage of method development, the chromatography was initially optimized by analyzing commercially available reference standards purchased as 1 mg powder. Since the analysis of a methanolic solution of the first acquired standard (Steraloids, Newport, RI, USA) revealed additional peaks highlighting an unsatisfactory purity grade, a second standard was purchased from another supplier (Merck KGaA, Darmstadt, Germany) which demonstrated satisfactory purity. Experimental results are reported in Supplementary Material Figure S1. With the chromatography and MS parameters optimized, the appropriate concentration range for observing PDG was determined. Since existing literature generally refers to 24-h urine samples rather than spot urine samples, this selection was based on a preliminary analysis of 200 spot urine samples recruited from the routine of the Clinical Biochemistry Laboratory of the City of Health and Science University Hospital of Turin. For this purpose, a 3-point calibration curve was established using three synthetic urine samples spiked with three different concentrations of PDG (0.1, 10, and 100 µg/mL). This curve was then used to analyze the 200 spot urine samples, and based on the measured concentrations, it was possible to select an appropriate calibration range (0.01–100 µg/mL) for the final method validation process.

3.2 | Method validation

3.2.1 | Calibration

For the calibration performed with the MVA software, the results of Levene test for the assessment of variance uniformity provided a p-value of 5.3×10^{-15}. Since this is less than 5×10^{-2}, the null hypothesis of homoscedasticity of the system is rejected. The creation of

TABLE 2 Results obtained from MVA software for the calibration evaluation.

Heteroscedasticity testing		
Levene test	***p*-value**	**Outcome**
	5.31×10^{-15}	Heteroscedastic
Weight selection		
Variance evaluation for weight selection	No weight	8.92×10^{-3}
	$1/x$	1.33×10^{-10}
	$1/x^2$	1.83×10^{-17}
Calibration model		
Mandel test	***F* Mandel**	***F* critic**
	11.35	3.98
Models residuals statical comparison	***p*-value (*t*-test)**	***p*-value (*F*-test)**
	1	0.26
Shapiro–Wilk test	**Test statistic**	***p*-value**
	0.97	0.10

Abbreviation: MVA, Methods Validation App.

regressions without and with weighting factors of $1/x$ and $1/x^2$ highlighted $1/x^2$ as the weighting factor generating the least variance. Additionally, the result of the Mandel test and the corresponding *F*-value are reported. If the *F*-value is greater than the critical *F*-value for the degrees of freedom of the system (critical *F*-value = 3.98), it indicates a rejection of the null hypothesis. This means that the explained variance differs significantly between the two models with the addition of the quadratic term, leading to the choice of using a second-degree equation.

The Mandel test suggests the use of a quadratic model for calibration, but following the application of *t*-tests and an *F*-test on the calibration residuals of both models using MVA, it was determined that the residuals do not differ significantly. This led to the choice of the less complex model, namely, the linear one. Additionally, a Shapiro–Wilk test was applied to assess the normality of the residuals. The results obtained for the calibration evaluation are reported in Table 2.

Figure 1 presents the calibration curve for the analyte under examination and its corresponding coefficient of determination R^2.

3.2.2 | Accuracy

Table 3 presents the results for the evaluation of intra-day and inter-day accuracy of the models for the analyte under examination in terms of bias%. It can be observed that the values obtained for both intra-day and inter-day evaluations show variations that follow the same trend across same calibration levels in different series of analysis. Additionally, all the values obtained are below 15%, allowing us to affirm the reliability of the values obtained for the method under examination.

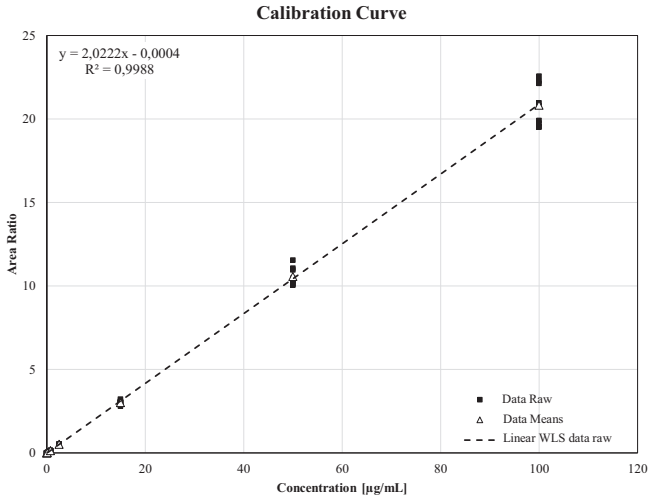

FIGURE 1 Calibration curve for PDG and corresponding coefficient of determination R^2. PDG, pregnanediol 3-glucuronide.

TABLE 3 Results for the evaluation of intra-day and inter-day accuracy of the models for PDG.

Concentration (µg/mL)	Bias intra-day (%)	Bias inter-day (%)
0.01	−0.12	−0.24
0.025	0.93	1.07
0.10	−1.95	−1.74
0.75	−4.60	−4.36
2.50	2.12	2.38
15.0	0.29	0.39
50.0	3.35	3.37
100.0	0.13	−0.35

Abbreviation: PDG, pregnanediol 3-glucuronide.

3.2.3 | Precision

Below, in Table 4, the results for the evaluation of intra-day and inter-day precision of the models for the analyte under study are presented in terms of CV%. The level 1 calibrator shows a higher CV% compared to the subsequent calibration levels; however, all results obtained for the different calibration levels are below 15%, indicating that the chosen model allows for reproducible estimation of the analyte concentrations.

3.2.4 | LOD and LLOQ

Thanks to the use of MVA, a LOD value of 0.005 µg/mL was obtained. The LLOQ, defined as the lowest tested concentration level for which intra- and inter-day precision and accuracy values lower than 20% were measured, is 0.01 µg/mL.

TABLE 4 Results for the evaluation of intra-day and inter-day precision of the models for PDG.

Concentration (μg/mL)	CV intra-day (%)	CV inter-day (%)
0.01	9.03	10.29
0.025	3.15	2.98
0.10	3.52	3.76
0.75	0.79	1.23
2.50	2.80	3.29
15.0	3.00	3.81
50.0	2.84	5.98
100.0	4.03	8.12

Abbreviations: CV, coefficient of variation; PDG, pregnanediol 3-glucuronide.

3.2.5 | Matrix effect

The comparation of the PDG peak area in urine sample to that in a methanolic solution, both with the same PDG concentration (50 μg/mL), allowed to evaluate the matrix effect. Each type of sample analyzed in triplicate revealed a slightly ion enhancement with matrix effect of 102.3% considered negligible. Additionally, the ratio of the PDG peak area in urine samples to that in synthetic urine samples was used to evaluate whether synthetic urine could serve as a good approximation of the real urine matrix. The observed matrix effect of 100.7% demonstrated no significant differences between the two matrices, confirming the suitability of synthetic urine for the preparation of calibration and validation samples.

3.2.6 | Selectivity

Two MRM transitions were selected for the target analyte to establish the initial level of selectivity for the method. Examination of MRM chromatograms of the 24 negative synthetic urine samples fortified with steroid glucuronide in the PDG elution region revealed no significant interferences (<20% LLOQ), except for the synthetic urines spiked with 17OH-pregnenolone glucuronide and EG, which exhibited peaks on the MRM transition of PDG but at a different retention time. The obtained chromatograms are presented in Supplementary Material Figure S2. Furthermore, the MS/MS spectra obtained from the eight real urine samples (four females and four males) analyzed in "Product Ion Scan" were compared with those from the analysis of a Level 5 calibration sample. Both real urine samples and calibration samples were also analyzed using the developed method. The measured fragment ratios, calculated by dividing the peak area obtained in the two MRM transitions, were compared between the female and male samples and the calibration samples. The results were within acceptable criteria (data presented in Supplementary Material Table S3).

3.2.7 | Carry-over

Negligible carry-over effect was observed for the target analyte by analyzing three diluted synthetic urine samples immediately after the most concentrated calibration sample (Level 8), with a value of 0.002% for the first diluted sample and 0.0% for the subsequent two diluted samples.

3.2.8 | Robustness

Despite the different operators, the different mobile phases prepared and used, and the two different analytical LC columns employed during the three days of analysis as a part of the method validation protocol, the results obtained permitted us to confirm that the developed method is robust within the linearity range for the analyzed compound.

3.2.9 | Stability

The stability of the extracted samples was assessed by storing three QC aliquots from the first validation series (day 0) at 4°C and reinjecting them 4 days later (day 4). Similarly, aliquots of the second validation series were stored at 4°C and reinjected 7 days later (day 7). The concentrations of the QC samples extracted on day 1 were compared to those stored for 4 and 7 days at 4°C. The quantification results, presented in Table 5, show concentration differences lower than 15% for all analyzed QCs. This finding demonstrates that the prepared compounds are stable in collection plates for at least 7 days at 4°C, which is a useful information for clinical laboratories which can now prepare and analyze samples in a 1-week period.

Furthermore, to evaluate the possibility of reusing a prepared calibration curve for multiple analytical batches, the four replicates of QC (low, medium, high) samples prepared during the first validation series were quantified using the calibration curve samples prepared on the same day and the calculated values were used as a reference (day 0). Four replicates of QC samples from the second validation series were processed using data from the calibration curve of day 0, which had been prepared and analyzed 4 days earlier (day 4). Additionally, four replicates of QC samples from the third validation series were processed using data from the day 0 calibration curve, which had been prepared and analyzed 7 days earlier (day 7). The concentrations of QCs prepared on days 4 and 7 were compared to those from day 0, and no concentration differences greater than 15% were observed for any of the detected target analytes. A summary of the stability assessment results is presented in Table 6.

3.3 | Real sample application

Regarding the application to real urine samples in the study, two stages were followed. For the first stage, carried out on seven healthy

TABLE 5 Stability of prepared samples stored for 3 and 7 days at 4°C after the preparation.

		Concentration (µg/mL)				
	Replicate	Day 0	Day 3	Recovery (%)	Day 7	Recovery (%)
QC low	1	0.611	0.588	96.2	0.606	99.3
	2	0.645	0.655	101.6	0.609	94.5
	3	0.628	0.662	105.4	0.612	97.4
	4	0.601	0.644	107.1	0.6	99.9
QC med	1	9.592	9.186	95.8	9.561	99.7
	2	9.519	9.154	96.2	9.132	95.9
	3	9.298	9.271	99.7	9.5	102.2
	4	9.817	9.612	97.9	9.44	96.2
QC high	1	65.434	69.519	106.2	66.407	101.5
	2	61.298	64.303	104.9	59.579	97.2
	3	63.145	67.009	106.1	61.425	97.3
	4	65.595	69.74	106.3	63.978	97.5

Abbreviation: QC, quality control.

TABLE 6 Stability of calibration 3 and 7 days after sample preparation.

		Concentration (µg/mL)				
	Replicate	Day 0	Day 3	Recovery (%)	Day 6	Recovery (%)
QC low	1	0.611	0.595	97.4	0.551	90.2
	2	0.645	0.603	93.5	0.634	98.4
	3	0.628	0.608	96.9	0.66	105.1
	4	0.601	0.592	98.5	0.629	104.7
QC med	1	9.592	8.412	87.7	9.254	96.5
	2	9.519	8.383	88.1	9.07	95.3
	3	9.298	8.49	91.3	8.954	96.3
	4	9.817	8.801	89.7	9.434	96.1
QC high	1	65.434	63.641	97.3	70.714	108.1
	2	61.298	58.867	96,00	64.485	105.2
	3	63.145	61.344	97.1	66.646	105.5
	4	65.595	63.844	97.3	70.9	108.1

Abbreviation: QC, quality control.

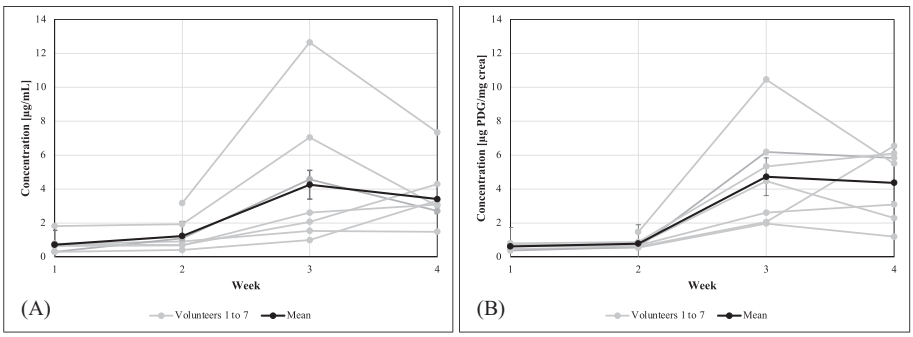

FIGURE 2 Monitoring of measured PDG urinary concentrations (a) and their values corrected for urinary creatinine (b) in 7 healthy female volunteers at 4 time points across a menstrual cycle. PDG, pregnanediol 3-glucuronide.

volunteers in Figure 2(a) are presented the PDG urinary concentrations measured in the urine samples collected during the 4 weeks of the study. The graph indicates that valid concentrations determinations were obtained for each subject. Specifically, the individual curves show that from the second week (day 14, onset of ovulation), there is a slight increase in concentration, peaking around the third week. This pattern aligns with the normal hormonal fluctuations of PDG, confirming ovulation. However, not all curves show PDG peaks

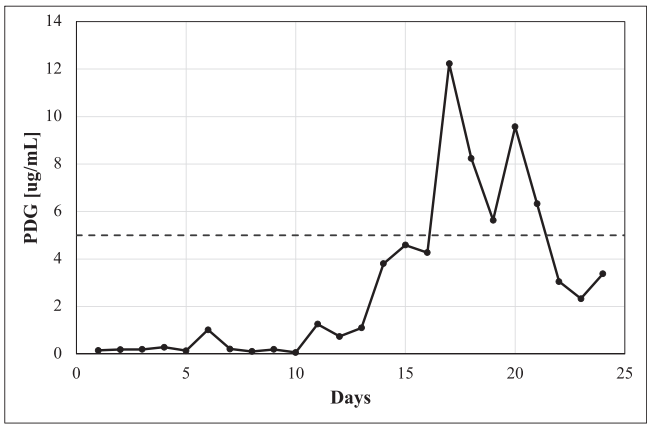

FIGURE 3 Longitudinal monitoring of PDG urinary concentrations across an entire menstrual cycle. PDG, pregnanediol 3-glucuronide.

above the 5 μg/mL threshold commonly cited in the literature to confirm ovulation, which may be attributed to inter-individual variability and the timing of sample collection.

In addition, to account for variations in urine sample volume and normalize PDG results, urinary creatinine was measured using a specific enzyme assay. Figure 2(b) shows the graph of PDG normalized for creatinine in each sample. The graph indicates that although creatinine provides a slight improvement in terms of comparability of different values between individuals, no significant differences are observed with or without creatinine adjustment. This outcome suggests that creatinine measurement may be unnecessary, resulting in significant time and cost savings associated with the enzymatic analysis.

Once the method for the determining PDG was proven suitable for real urine sample analysis, the second stage involved a longitudinal study analyzing samples from a 34-year-old woman without hormonal dysfunction. In this case, the subject's menstrual cycle is shortened to 25 days. Figure 3 shows the longitudinal monitoring profile, revealing a clear increase in PDG concentration from day 14 (as in the previous case shown in Figure 2), with two peaks around days 17 and 20, corresponding to the period of highest PDG levels. Despite the short duration of the menstrual cycle, the peaks occur 5–8 days before the next menstruation, confirming the physiology of the phenomenon. This graph highlights ovulation, as values above the 5 μg/mL threshold proposed in literature were observed for three consecutive days (Leiva et al., 2019). The day-by-day monitoring of the menstrual cycle allows for a more accurate profile in determining ovulation trends, thus confirming the validity of the method for monitoring the occurrence of ovulation.

4 | CONCLUSIONS

In this study, a rapid and efficient UHPLC–MS/MS method for PDG determination in spot urine samples for ovulation detection was developed and validated. The presented analytical platform proved suitable for PDG measurement in 7 μL of spot urine samples, demonstrating excellent characteristics in terms of MS analysis by providing good precision, accuracy, specificity, and sensitivity. The method allows for a very small sample volume and provides a reliable rapidity with a chromatographic run time of only 6 min. Additionally, the absence of a sample extraction procedure, with a simple dilute-shoot step, reduces analysis time and makes the method attractive to clinical chemistry laboratories due to its adaptability to automation. While the absence of a pre-analytical extraction step may reduce analytical costs, it could potentially decrease column utilization rate. However, in this study, 100× diluted urine samples were used, potentially reducing column wear and tear. Such issues can be mitigated through the use of a pre-column, which can extend column life. Based on the analysis performed and the results obtained, the method has proven to be highly valid for analyzing real samples. Its speed and efficiency make it particularly suitable for assessing larger patient cohorts. Furthermore, there is potential for future applications that could explore additional markers relevant to ovulation determination, such as EG. The concurrent monitoring of EG, another ovarian steroid, alongside PDG concentration determination, could offer further confirmation of ovulation.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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