A Validated Method for the Simultaneous Determination of Oxytocin and Cortisol in Human Saliva

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Abstract: Oxytocin and cortisol (OXY and CORT) are hormones related to stress, cognitive, and social behaviors. Their detection is relevant to epidemiological studies aimed at investigating the effects of stressor factors on human life. The aim of this study was to develop and validate an assay for the measurement of OXY and CORT in saliva samples using liquid chromatography/tandem mass spectrometry (LC-MS/MS) in the presence of deuterated analogs. A 500 mL aliquot of oral fluid, obtained by the centrifugation of a chewed swab, was purified by solid-phase extraction. Analytes were then separated using C18 reversed-phase chromatography, subjected to positive electrospray ionization, and then quantified using a triple-quadrupole mass detector in multiple-reaction monitoring mode. The limits of quantification and the linear dynamic ranges were $2.0 \times 10^{-3}$ and 0.5 nmol/L, and up to $1.0 \times 10^{-1}$ and 20 nmol/L for OXY and CORT, respectively. Inter- and intra-run precision, expressed as relative standard deviation, was <7%, and accuracy was within 93–104% of the theoretical concentrations. The evaluation of matrix effects showed that the use of internal standards controlled sources of bias. The high sensitivity of the method allowed the quantification of OXY and CORT in the salivary samples of both adults and children: levels of CORT ranged from 0.6 to 18.5 nmol/L, while OXY levels were two orders of magnitude lower (from $1.7 \times 10^{-3}$ to $1.1 \times 10^{-2}$ nmol/L). To our knowledge, this is the first method that can analyze, in the same chromatographic run, both hormones in saliva samples.

Keywords: oxytocin; cortisol; LC-MS/MS; method validation; saliva

1. Introduction

Oxytocin (OXY) is a hormone synthesized primarily by neurons in the supraoptic and paraventricular nuclei of the hypothalamus, and is released from the posterior pituitary; it is known for its evolutionary and pharmacological actions [1]. Centrally, OXY is associated with maternal, sexual, social, and stress-related behaviors, while peripherally it is most associated with smooth muscle contraction in the female reproductive tract. Moreover, there is increasing evidence that OXY also has diverse effects on fat and bone tissue, as well as on the pancreas, heart, and kidney [2,3]. Besides its well-known role in pregnancy, parturition, and lactation, recently OXY has been related to prosocial behaviors such as trust and affiliation between individuals, as well as reductions in fear and anxiety [4]. Based on its crucial role in social support and stress regulation, OXY contributes to maintaining mental health. For instance, OXY administration increases positive communication and attenuates stressful responsiveness to both social and physical stress by reducing the release of stress hormones [5,6]. To better understand these and other roles of OXY in non-pregnant/non-lactating/non-medicated individuals, accurate quantification methods are urgently required.
Cortisol (CORT) is a steroid hormone produced by the adrenal glands that sit atop the kidneys. It is released in response to stress and low levels of glucocorticoids in the blood. The primary functions of CORT are to increase blood sugar through gluconeogenesis, suppress the immune system, and aid in fat, protein, and carbohydrate metabolism. It also activates anti-stress and anti-inflammatory signaling pathways [4]. Currently, the determination of CORT levels is proposed in clinical practice to aid the diagnosis of altered hypothalamic–pituitary–adrenal axis secretion, which occurs in cases of Cushing’s syndrome or subclinical hypercortisolism [7]. CORT is also assessed as a stress hormone in epidemiological research in occupational and general population settings [8–10].

Oral fluid represents an attractive specimen for human biomonitoring as it does not require an invasive collection, sampling can be managed autonomously by the investigated subjects simply by chewing a swab, and it can be performed as many times as required. In this type of matrix, OXY and CORT are commonly measured by different methods, often based on enzyme immunoassays, including radio immunoassays (EIA/ELISA, RIA) [11–15]; however, these methods may lack the specificity and sensitivity needed to accurately quantitate the very low native levels of these hormones in non-pregnant/non-lactating/non-medicated mammals, and/or they use dangerous radioisotopes. Solid-phase extraction coupled with liquid chromatography–tandem mass spectrometry (LC-MS/MS) is very effective in removing matrix interferences and provides the required selectivity and sensitivity. For CORT measurement, several methods using LC-MS/MS are reported in the literature (for example, [16–18]), while there are a few for measuring OXY at basal levels [19].

The aim of the present work was the development and validation of an assay for the simultaneous quantification of OXY and CORT in saliva useful for epidemiological studies. The assay involves the preparation of the sample by solid-phase extraction, followed by LC-MS/MS analysis in the presence of isotopically labelled internal standards. To evaluate its suitability, the method was applied to measure OXY and CORT in the saliva of healthy individuals of different ages, including adults and children.

2. Materials and Methods

2.1. Chemicals

Analytical standards of cortisol and oxytocin acetate (both at a purity ≥ 98%) were purchased from Sigma-Aldrich (Milan, Italy). Cortisol-D₄ (9,11,12,12-D₄ with an isotopic purity ≥ 98%; CORT-IS) and Oxytocin-D₅ trifluoroacetate (leucine-5,5,5-D₃ and glycine-2,2-D₂ with an isotopic purity ≥ 98%; OXY-IS) were purchased from Sigma-Aldrich (Milan, Italy) and used for the preparation of the internal standard (IS) solution. For the mobile phases, standard solutions, assay optimization, and sample preparation, methanol (MeOH), acetonitrile (CH₃CN), formic acid, and phosphoric acid (all LC-MS/MS-grade, Sigma-Aldrich, Milan, Italy) were used. A Milli-Q Plus ultrapure water system (Millipore, Milford, MA, USA) was used for purifying water.

2.2. Standard, Calibration, and Quality Control Solutions

Standard solutions at concentrations of 2.5 and 2.5 × 10⁻¹ nmol/L for OXY and 500 and 50 for CORT were prepared in MeOH. The internal standard (IS) solution was prepared from pure OXY-IS and CORT-IS, and dissolved in methanol to final concentrations of 9.9 × 10⁻¹ and 250 nmol/L, respectively. Standard and IS solutions were stored at −20°C in the dark in polypropylene tubes. Under these conditions, the solutions remained stable for up to 6 months.

To assess the linearity of the method, calibration standard solutions containing OXY (2.0 × 10⁻³, 5.0 × 10⁻³, 7.4 × 10⁻³, 9.9 × 10⁻³, 2.5 × 10⁻², 5.0 × 10⁻², 7.4 × 10⁻², and 1.0 × 10⁻¹ nmol/L) and CORT (0.5, 1.0, 1.5, 2.0, 5.0, 10, 15, and 20 nmol/L) were prepared by adding suitable amounts of standard solutions to water. An unspiked sample of water was used as a blank. This approach was acceptable based on the evaluation of the matrix effect. Salivary samples with certified CORT concentrations (0.81 and 7.33 nmol/L)
(Chromsystems, Munich, Germany) were used as quality controls (QCs) after the addition of a suitable amount of OXY (to the final concentration of $4.2 \times 10^{-3}$ and $3.6 \times 10^{-2}$ nmol/L) to obtain low- and high-QC samples. Salivary samples from healthy volunteers were used as a matrix for the matrix-effect experiment.

During the analytical procedure, the IS solution was added to each sample (calibration solutions, QCs, and unknown samples) to a final concentration of $4.0 \times 10^{-2}$ and 10 nmol/L for OXY-IS and CORT-IS, respectively.

2.3. Equipment

For sample purification, a solid-phase extraction cartridge (SPE, Oasis HLB, 30 mg × 1 mL, Waters, Vimodrone, Milan, Italy) was used. Samples were concentrated to dryness under a gentle flow of nitrogen using a dry block (Reacti-Vap Pierce, Milan, Italy). A high-performance liquid chromatograph (Agilent Technologies 1260, Cernusco sul Naviglio, Italy) interfaced with a hybrid triple-quadrupole/linear ion trap mass spectrometer (QTRAP 5500; Sciex, Monza, Italy) equipped with an electrospray ionization source (ESI) was used for the analysis.

2.4. LC-MS/MS Analysis

A Hypersil Gold column (50 mm length, 2.1 mm i.d., 3 mm particle size, Thermo Scientific, Rodano, Italy) kept at 35 °C was used for the separation, and a linear gradient with an aqueous solution of ammonium formate (5 mM) with 0.1% formic acid (phase A) and ammonium formate (5 mM) with 0.1% formic acid in MeOH (phase B), flowing at 400 µL/min, was applied. The gradient consisted of the following steps: step 1, isocratic with 0% of phase B (0–1 min); step 2, from 0% to 100% of phase B (1–6 min); step 3, isocratic with 100% of phase B (6–7 min); step 4, from 100% to 0% of phase B (7–8 min); step 5, isocratic with 0% of phase B (8–13 min). Multiple-reaction monitoring (MRM) mode was used to acquire signals, with a dwell time of 50 ms. The ESI source was set in positive ionization mode and the principal optimized parameters were as follows: gas pressure 1: 30 psi; gas pressure 2: 40 psi; curtain gas pressure: 35 psi; heater temperature: 450 °C; declustering potential: 70 V; input potential: 10 V. The MRM ion transitions recorded were the two most intense for each native analyte; the most intense was chosen as a quantifier and the other as a qualifier, in line with the most intense for each isotopically labeled standard (Table 1). To set up the method and analysis batches, Analyst® software (version number 3.3, Sciex, Monza, Italy) was used, and MultiQuant™ software (version number 3.0.3, Sciex, Monza, Italy) was used for quantification.

2.5. Sample Collection and Preparation

Saliva samples were autonomously collected by healthy study subjects using Salivette® devices, according to the manufacturer’s instructions. Once in the laboratory, saliva was extracted from each swab by centrifugation (5 min at 3000 rpm), and samples were stored in Salivette® polyethylene tubes at −20 °C in the dark until the analysis.

Before the analysis, samples (calibrators, QCs, and unknown samples) were diluted (1:1) with a solution of 4% phosphoric acid in water and added to 20 µL of IS solution. After this process, they were purified with SPE cartridges. This procedure involves several steps: 1 mL of CH$_3$CN and then 1 mL of 1% phosphoric acid in water were used to conditionate the cartridge; after that, 1 mL of the diluted sample was loaded into the cartridge and subsequently washed with 1 mL of a solution of 1% phosphoric acid in water; finally, 1 mL of MeOH was used to elute the analytes. A gentle stream of nitrogen, with the heating block set at 37 °C, was employed to evaporate the eluate. The dried samples were reconstituted in 100 µL of a mixture of the mobile phases (70 A:30 B), stirred vigorously by vortexing, and transferred into a glass insert; a volume of 40 µL was injected into the LC-MS/MS for analysis.
Table 1. Principal LC-MS/MS parameters for the analysis of OXY and CORT and their internal standards. CAS number, molecular structures, MRM transitions (Q1 and Q3) for quantifier and qualifier ions, collision energies (CEs) and chromatographic retention times are given.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>CAS Number</th>
<th>Molecular Structure</th>
<th>Description</th>
<th>Q1 Mass (Da)</th>
<th>Q3 Mass (Da)</th>
<th>CE (V)</th>
<th>Retention Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxytocin acetate (OXY)</td>
<td>6233-83-6</td>
<td><img src="image" alt="Oxytocin Acetate" /></td>
<td>Quantifier</td>
<td>1007</td>
<td>723</td>
<td>30</td>
<td>6.76</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Oxytocin-D₅ trifluoroacetate</td>
<td>50-56-6</td>
<td><img src="image" alt="Oxytocin-D₅ Trifluoroacetate" /></td>
<td>IS</td>
<td>1012</td>
<td>723</td>
<td>30</td>
<td>6.75</td>
</tr>
<tr>
<td>(leucine-5,5,5-D₃, and glycine-2,2-D₂) (OXY-IS)</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Cortisol (CORT)</td>
<td>50-23-7</td>
<td><img src="image" alt="Cortisol" /></td>
<td>Quantifier</td>
<td>363</td>
<td>121</td>
<td>33</td>
<td>7.71</td>
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<td></td>
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</tr>
<tr>
<td>Cortisol-D₄ (9,11,12, 12-D₄)</td>
<td>73565-87-4</td>
<td><img src="image" alt="Cortisol-D₄" /></td>
<td>IS</td>
<td>367</td>
<td>121</td>
<td>33</td>
<td>7.70</td>
</tr>
</tbody>
</table>
2.6. Set Up of the Analytical Sequence

As part of the routine analysis, calibration solutions and QCs were run with each set of unknown samples. A typical analytical sequence consisted of the set of calibration solutions, followed by unknown samples with a QC sample (low or high QC) as every tenth unknown sample, followed again by the calibration solutions.

2.7. Optimization of the Method
2.7.1. SPE Extraction and Purification

Oasis HLB cartridges were chosen based on the previous use of this stationary phase for OXY in plasma as a matrix [20]. For the optimization of the extraction procedure and the recovery evaluation, low- and high-QC samples of saliva (n = 3 for each level) were analyzed in two different batches. In the optimization experiment, after the sample loading, all fractions were collected and analyzed to test the possible leaching of analytes during the washing step and to better optimize the extraction fraction. The recovery was calculated as the percent ratio between the chromatographic signals of purified extract versus the signals of CORT and OXY in water that were directly injected into the chromatographic system.

2.7.2. Selection of Chromatographic Condition and MS/MS Analysis

Different linear gradient programs, as well as different organic solvents (MeOH or CH$_3$CN) and additions of acid (no acid; 0.1% formic acid; or 0.5% formic acid), were tested to improve the peak separation, peak shape, retention times, and signal-to-noise ratio. A combination of manual and automatic tuning with a standard solution infused in water (0.1 mg/L) was used to optimize the MS/MS working conditions, including ESI and ionization parameters, MRM transitions, and collision energies.

2.8. Assay Validation
2.8.1. Calibration Curve, Limits of Detection and Quantification, Carryover, Mid-Term Stability, Precision, Accuracy, Matrix Effect, and Curve in Saliva Versus Water

The calibration curve was prepared with a blank and seven non-zero calibration solutions, covering the expected concentration range. Least-squares linear regression analysis was applied to interpolate the data pairs; the value of $y$ corresponds to the ratio of the areas of the chromatographic peaks of the analyte and the corresponding IS, and $x$ is the analyte concentration in nmol/L. For method validation, ten replicates of each calibration level were analyzed.

To calculate the limit of detection (LOD) and limit of quantification (LOQ) of the assay, the following expression was used:

$$\text{LOD/LOQ} = \frac{(3/5SE_q + q)}{m}$$

where $SE_q$ is the standard error of the intercept $q$, and $m$ is the slope of the linear regression. To verify if the obtained results met the requirements of the US FDA recommendations and the acceptance criteria for bioanalytical method validation for LOQ assessments, precision (expressed as the coefficient of variation, %RSD) and accuracy (calculated as %Theoretical) were calculated at the LOQ [21].

To test the carryover effect, a water sample was run immediately after the analysis of the highest calibration solution.

To calculate the mid-term stability, the variability of the slopes of the calibration curves (n = 10) over a six-month period was evaluated, expressed as %RSD$_{\text{slope}}$.

By running the low- and high-QC solutions three times on the same day, intra- and inter-day precision and accuracy were calculated on six different days over a period of 6 months. Precision was expressed as the relative standard deviation (%RSD). Accuracy was calculated as the percent ratio between the concentrations calculated from the calibration curves and the theoretical concentrations (%Theoretical).
For the matrix-effect experiment, saliva samples from six different donors were used. Calibration curves were obtained with the procedure described in Section 2.2. The inter-matrix slope range (\(\%R_{\text{slope}}\)) was calculated by the following formula [22]:

\[
\%R_{\text{slope}} = \frac{(\text{Maximum}_{\text{slope}} - \text{Minimum}_{\text{slope}})}{\text{Minimum}_{\text{slope}}} \times 100
\]

where \(\text{Maximum}_{\text{slope}}\) and \(\text{Minimum}_{\text{slope}}\) are the highest and lowest slope values of the calibration curves obtained using saliva from the different subjects. In order to compare results, all computations were performed both with and without the IS.

In the specificity test, an aqueous solution of prednisolone and prednisone at final concentrations of 400 nmol/L and 250 nmol/L, respectively, was used to simulate the maximum levels expected in human saliva following the administration of 80 mg prednisolone (the maximum therapeutic dose) to salivary samples from three healthy subjects that did not use this medicine.

To investigate the suitability of the calibration curves prepared in water for the quantification of the saliva samples, the levels of CORT and OXY in the matrix-effect solutions were back-calculated against the calibration curves obtained in water. After subtracting the background noise, the accuracy (\(\%\text{Theoretical}\)) and precision (\(\%\text{RSD}\)) values were evaluated. Moreover, the slopes of the calibration curves determined in water versus oral fluids were compared, and a percent of relative difference (\(\%\Delta\)) was calculated.

2.8.2. Method Comparison for CORT

For CORT, the present SPE LC-MS/MS method was compared with a TurboFlow LC-MS/MS method, previously set by our group and applied in the routine work in our lab [16]. Data obtained from the two methods were compared with a nonparametric Passing–Bablok regression analysis [23] and a Cusum test; the Bland–Altman approach was used to assess the differences between the two methods by plotting the relative difference between the two assays versus the mean concentration [24].

2.9. Method Application

In order to evaluate the applicability of the developed method to real samples, saliva samples collected from thirty-one convenient donors (11 males and 20 females; age ranging from 10 to 70 years) were analyzed. Due to the circadian behavior of CORT, oral fluid samples were collected at two different times of day: in the morning (around 8:00) and in the evening (around 23:00) on any workday. Samples (kept at \(-20^\circ\)C until the analysis) were collected, prepared, and analyzed as described above.

3. Results and Discussion

3.1. Optimization of the Method

3.1.1. SPE Extraction and Purification

In the optimization experiment, no analytes were found in the loading fraction or during the cartridge wash; the elution was performed with three consecutive steps, using 1 mL MeOH in each. During the first elution, the recovery was 100% and 98% for OXY and CORT. On this basis, the final procedure included a single elution with 1 mL MeOH, allowing the complete elution of the analytes and avoiding solvent waste.

The recovery from saliva ranged from 80% to 99%, with a lower recovery for CORT (from 80% to 91%) compared to OXY (from 86% to 99%); the recovery of ISs was similar to that of the respective analytes. Indeed, the correction of the signal of the analytes by the ISs resulted in a recovery close to 100% (97% and 101% for CORT and OXY, respectively), confirming the importance of using isotopically labelled analogs to ensure reliable analytical performance.
3.1.2. Selection of Chromatographic Condition and MS/MS Analysis

Figure 1 shows an example chromatogram of the quantifier ions of OXY, CORT, and their internal standards (OXY-IS and CORT-IS) in a sample of saliva from a donor. The retention time was about 6.7 min for OXY and OXY-IS, and 7.7 min for CORT and CORT-IS; these are long enough to overcome the matrix interferences typically present at short retention times and thus enhance sensitivity, but short enough to allow a rapid total run time (less than 15 min per sample).

Figure 1. Chromatogram of the quantifier ions for OXY (green line), OXY-IS (grey line), CORT (blue line), and CORT-IS (red line) in real saliva sample (2.2 × 10⁻³ and 18.5 nmol/L for OXY and CORT, respectively).

In Figure 2, the quantifier ion chromatograms of OXY (a), CORT (b), and their internal standards are shown in a blank sample, in a sample containing the analytes at the LOQ level, and in a saliva sample from a donor. The chromatographic signals for both OXY and CORT are clearly distinguishable from the noise, allowing us to achieve LOQs low enough to quantify most of the saliva samples.

To improve the peak separation, peak shape, retention times, and signal-to-noise ratio, different chromatographic conditions were tested. Finally, a diluted buffer (5 mM of ammonium formate) with a small percentage of formic acid, prepared in both water and MeOH, was found to be the most effective mobile phase.

The principal tuning parameters and chromatographic conditions are reported in Table 1. Signals were registered in positive ionization mode; for each chemical, the transitions producing the most abundant ion were chosen for quantification (m/z 1007 → 723 for OXY; m/z 1012 → 723 for OXY-IS; m/z 363 → 121 for CORT; m/z 367 → 121 for CORT-IS).
3.2. Assay Validation

3.2.1. Calibration Curve, Limits of Detection and Quantification, Carryover, Mid-Term Stability, Precision, Accuracy, Matrix Effect, and Curve in Oral Fluid Versus Water

A summary of the validation parameters is reported in Table 2. Acceptable linearity was found for both analytes, with coefficients of determination ($R^2$) higher than 0.992. The LOQs were $2.0 \times 10^{-3}$ and 0.5 nmol/L for OXY and CORT, respectively. These LOQs are in line with or better than those previously reported for both analytes [16,19,25]. At the LOQ, precision was 7.4% for OXY and 3.0% for CORT, while accuracy was 112% for OXY and 110% for CORT. No carryover was evident. For both analytes, the mid-term stability of the calibration curve was acceptable, with %RSD_{slope} up to 2.5%, i.e., within the range of intra-day precision. The inter- and intra-run precision and accuracy of the assay met the US FDA requirements for the validation of bioanalytical methods (precision estimated as %RSD < 10%; accuracy between 93 and 104% of the theoretical concentrations) [21].
Table 2. Calibration curve results with limit of detection (LOD) and limit of quantification (LOQ), inter-matrix slope range (%RSD$\text{slope}$), matrix effect (%R$\text{slope}$ and %Matrix$\text{relative}$), comparison between water and matrix calibration curves (%RSD, %Theoretical and %$\Delta$), and precision and accuracy of the assay calculated for low- and high-QC samples.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Concentration Range (nmol/L)</th>
<th>LOD</th>
<th>LOQ</th>
<th>%RSD at LOQ Level</th>
<th>%Theoretical at LOQ Level</th>
<th>%RSD$\text{slope}$ without IS</th>
<th>%R$\text{slope}$ with IS</th>
<th>%RSD$\text{slope}$ without IS</th>
<th>%R$\text{slope}$ with IS</th>
<th>%Theoretical</th>
<th>%RSD</th>
<th>%Theoretical</th>
</tr>
</thead>
<tbody>
<tr>
<td>OXY</td>
<td>$2.0 \times 10^{-3}$-$7.4 \times 10^{-4}$</td>
<td>$9.9 \times 10^{-4}$</td>
<td>$2.0 \times 10^{-3}$</td>
<td>7.4</td>
<td>112</td>
<td>0.9</td>
<td>31.9</td>
<td>2.4</td>
<td>4.0</td>
<td>104</td>
<td>1.8</td>
<td></td>
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<tr>
<td>CORT</td>
<td>0.5–15</td>
<td>0.3</td>
<td>0.5</td>
<td>3.0</td>
<td>110</td>
<td>2.5</td>
<td>33.8</td>
<td>2.6</td>
<td>3.4</td>
<td>94</td>
<td>0.8</td>
<td></td>
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</table>

<table>
<thead>
<tr>
<th>QC</th>
<th>%RSD (Min–Max)</th>
<th>%Theoretical (Min–Max)</th>
<th>%RSD</th>
<th>%Theoretical</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1–6</td>
<td>n = 3</td>
<td>Overall n = 18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>2.3–6.6</td>
<td>93–102</td>
<td>5.0</td>
<td>99</td>
</tr>
<tr>
<td>High</td>
<td>0.3–1.6</td>
<td>100–104</td>
<td>1.4</td>
<td>102</td>
</tr>
<tr>
<td>Low</td>
<td>0.6–5.6</td>
<td>98–104</td>
<td>4.6</td>
<td>101</td>
</tr>
<tr>
<td>High</td>
<td>0.4–5.4</td>
<td>94–100</td>
<td>3.7</td>
<td>98</td>
</tr>
</tbody>
</table>
For both OXY and CORT, a significant matrix effect was found; this was, however, completely overcome using the IS correction. The %R_slope values, representing the maximum difference in the slope values obtained using matrices from different individuals, were 31.9% and 33.8% without the ISs for OXY and CORT, respectively. These were reduced to below 2.6% with IS correction. This result shows that the use of isotopically labelled ISs is effective in reducing the existing matrix effect and in maintaining acceptable analytical performance, as previously reported [16,19]. In the specificity test, no interference from prednisone or prednisolone was observed in the quantification of OXY and CORT.

The matrix-effect solutions that were back-calculated using calibration curves obtained in water yielded results with high accuracy (90–113%) and precision (≤8.5%). These results, together with the comparability of the slopes of the calibration curves prepared in oral fluids and in water (%Δ ≤ 1.8%), demonstrate that the use of water calibration curves does not introduce a quantification bias in the assay. A similar result was previously reported for the determination of hormones in different matrix samples [16,26].

### 3.2.2. Method Comparison for CORT

A comparison between the present method and the method used by our lab in routine work is shown in Figure 3. For the Passing–Bablok regression, data pairs are presented in scatter plots, with the TurboFlow LC-MS/MS results plotted along the x axis and the SPE LC-MS/MS results plotted along the y axis (Figure 3a). A Bland–Altman plot was used to compare the two assays, with the difference% plotted on the y axis (Figure 3b) and the mean concentration of the two methods plotted on the x axis. The Passing–Bablok intercept of the linear regression was −0.018 (95% CI: −0.086–0.015, including zero) and the slope was 1.006 (95% CI: 0.99–1.03, including 1). The Cusum test showed a non-significant deviation from linearity (p > 0.05). These results indicate the absence of both constant and proportional biases in the present method. According to the Bland–Altman plot, a mean difference of 0.57% (from −6.72% to 7.86%) was found; the highest discrepancy was observed at the lowest concentrations.

![Figure 3](image-url)

**Figure 3.** Method comparison between salivary levels of CORT assessed by TurboFlow LC-MS/MS versus the present method of SPE LC-MS/MS. (a) Passing–Bablok correlation: the correlation lines are represented by dashed-red lines, while the dotted line indicates the line of identity. (b) Bland–Altman plot to identify relative differences: mean differences are represented by solid black lines and 95% limits of agreement are represented by dashed-red lines.

The comparison of the method for CORT indicates a negligible discrepancy between the TurboFlow LC-MS/MS and SPE LC-MS/MS assays.
3.3. Method Application

The results pertaining to OXY and CORT in the salivary samples of 31 donors are summarized in Table 3.

Table 3. Results of OXY and CORT (nmol/L) in salivary samples of individuals of the general population.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>TOT</th>
<th>Gender</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N = 31</td>
<td>N = 11</td>
<td>N = 20</td>
</tr>
<tr>
<td>OXY</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N° samples &gt; LOQ (%)</td>
<td>29 (94%)</td>
<td>10 (91%)</td>
<td>19 (95%)</td>
</tr>
<tr>
<td>Mean</td>
<td>3.4 \times 10^{-3}</td>
<td>2.7 \times 10^{-3}</td>
<td>3.7 \times 10^{-3}</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>2.0 \times 10^{-3}</td>
<td>9.6 \times 10^{-4}</td>
<td>2.3 \times 10^{-3}</td>
</tr>
<tr>
<td>Median</td>
<td>2.5 \times 10^{-3}</td>
<td>2.5 \times 10^{-3}</td>
<td>2.6 \times 10^{-3}</td>
</tr>
<tr>
<td>Min–Max</td>
<td>1.7 \times 10^{-3}–1.1 \times 10^{-2}</td>
<td>1.7 \times 10^{-3}–5.2 \times 10^{-3}</td>
<td>1.9 \times 10^{-3}–1.1 \times 10^{-2}</td>
</tr>
<tr>
<td>CORT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N° samples &gt; LOQ (%)</td>
<td>31 (100%)</td>
<td>31 (100%)</td>
<td>31 (100%)</td>
</tr>
<tr>
<td>Mean</td>
<td>4.8</td>
<td>4.7</td>
<td>4.8</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>4.8</td>
<td>3.4</td>
<td>5.6</td>
</tr>
<tr>
<td>Median</td>
<td>3.0</td>
<td>4.4</td>
<td>2.4</td>
</tr>
<tr>
<td>Min–Max</td>
<td>0.6–18.5</td>
<td>0.6–9.9</td>
<td>0.8–18.5</td>
</tr>
</tbody>
</table>

For OXY, two samples (94% of the analyzed samples) were in the range between LOD and LOQ (1.7 \times 10^{-3} and 1.9 \times 10^{-3} nmol/L), while for CORT, all samples were above the analytical LOQ.

Levels of CORT ranged from 0.6 to 18.5 nmol/L, while levels of OXY were two orders of magnitude lower (from 1.7 \times 10^{-3} to 1.1 \times 10^{-2} nmol/L). Indeed, sensitivity for OXY is a relevant issue because the physiological levels of this hormone are very low. For this reason, we developed the method with particular attention to the response of OXY. The LOQ achieved was very low (2.0 \times 10^{-3} nmol/L), allowing us to measure OXY in the large majority (>90%) of tested samples.

Samples were further divided by sex, sample time, and age based on approaches reported in the literature. OXY levels were indeed significantly different between sex, with means of 2.7 \times 10^{-3} and 3.7 \times 10^{-3} nmol/L in males and females, respectively (p = 0.03, see Figure 4). Otherwise, no significant difference was found for sampling time or age. CORT levels were higher in the morning than in the evening samples, with mean concentrations of 8.4 nmol/L and 1.5 nmol/L, respectively (p < 0.001); moreover, a tendency toward higher levels was also observed in children compared to adults, with mean concentrations of 6.0 and 4.4 nmol/L, respectively (p = 0.07). No differences were found for the sex category.

The higher level of OXY in females than in males is consistent with previously reported results. Such differences are attributed to the biological activity of OXY in smooth muscle contraction in the reproductive tract in women [2,3].

It is well known that CORT follows a circadian cycle, with the highest concentrations in the morning and minimum levels at night [27,28]; for this hormone, higher concentrations in children than in adults are also reported [29].

The OXY concentrations measured in the present study are comparable to those previously measured by LC-MS/MS [19,25], but much lower than those measured by immunoassays [30,31]. Similarly, for CORT levels, the values found in the present study are comparable to those measured by LC-MS/MS [16,17,32], but much lower than those measured by immunoassays [14,33]. This points to the low specificity of immunoassays.
Figure 4. Difference between salivary OXY concentrations in study subjects divided by sex.

4. Conclusions

To our knowledge, this is the first method that allows the simultaneous measurement of OXY and CORT in saliva. The method is based on LC-MS/MS and is validated according to international guidelines, using isotopically labelled internal standards to control sources of bias. Its good performance in terms of linearity, sensitivity, precision, and accuracy ensure its applicability in epidemiological studies for the quantification of OXY and CORT in salivary samples.

Author Contributions: Conceptualization, E.P., R.M., L.C., and S.F.; methodology, E.P. and R.M.; validation, E.P.; formal analysis, E.P.; investigation, E.P.; data curation, E.P.; writing—original draft preparation, E.P.; writing—review and editing, R.M., L.C., and S.F.; visualization, E.P.; supervision, S.F.; project administration, S.F. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki. Since ours is a purely observational study (non-pharmacological and non-profit), the approval of an ethics committee was not necessary according to Italian legislation (Decreto della Direzione Generale della Sanità n.11960 del 13.07.2004 relativo all’approvazione delle linee guida sugli Studi “Osservazionali” o “non interventistici”; Determinazione AIFA—20 marzo 2008. Linee guida per la classificazione e conduzione degli studi osservazionali sui farmaci. (GU n. 76 del 31-3-2008)). In addition, institutional review board approval was not required because the study comprises a routine activity focused on the possibility of introducing new technology and aligning different methods, for which no extra biological samples nor sensitive data were requested. However, for each patient entering the hospital, signed informed consent with a general agreement to the use of specimens for research is routinely collected.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study. Privacy was protected according to the Italian regulations in force (D.L.vo 196/2003 e le Linee Guida del Garante, Deliberazione n. 52 del 24/07/2008).

Data Availability Statement: The data presented in this study may be available upon reasonable request from the corresponding author. The data are not publicly available due to privacy and legal restrictions.

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Conflicts of Interest: The authors declare no conflicts of interest.
References


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