DEVELOPMENT OF A VALIDATED UV-VIS SPECTROPHOTOMETRIC METHOD TO QUANTIFY CORTISOL IN SALIVA SAMPLES OF YOUNG AND MIDDLE ADULTS

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ABSTRACT: Background: Cortisol quantification is important in clinical practice to measure periods of stress. The cortisol test is used to diagnose adrenal gland disorders. These include Cushing's syndrome, which causes the body to make too much cortisol, and Addison's disease, which causes the body to not make enough cortisol. **Objective:** In this work was to develop a validated UV/Vis spectrophotometric method to quantify cortisol in saliva samples from subjects whose ages ranged from 18 to 24 (25 women and 10 men) and ranged from 45 to 55 years old (8 women and 7 men). **Methods:** For the experimental study, a saliva sample was taken in the first hours of the morning (1.5 mL). The sample was homogenized and read at an absorbance of 280 nm using diluted saliva as a control and carrying out an extraction with ethanol and trichloroacetic acid to identify cortisol. **Results:** This method was linear (r = 0.9999) for an interval of 2.5 to 25 μg/mL, accurate, precise (variation coefficients of repeatability and reproducibility were less than 1.5%, andless than 2%, respectively). it is specific and with limits of detection and quantification of 0.019 μg/mL and 0.16 μg/mL. The concentration in young people was 52.58 ± 8.59 µg/dL (women) and 49.75 ± 7.65 µg/dL (men); in older adults 38.83 ± 2.82 µg/dL (women) and using this method 25.76 µg/dL (men). **Conclusion:** This method shows to be specific and sensitive with limits of detection and quantification of 0.019 μg/mL and 0.16 μg/mL, respectively. Cortisol average concentration found using this method was higher in young people (women= 52.58 ± 8.59 µg/dL and men= 49.75 ± 7.65 µg/dL) than in older adults (women= 38.83 ± 2.82 and men= 25.76 µg/dL).

KEYWORDS: Cortisol; Validation of analytical method; UV-vis Spectrophotometry; Hydrocortisone; Saliva samples.

INTRODUCTION

Cortisol is a lipophilic steroid that is dominated by the circadian cycle, which is secreted through the Hypothalamic- Pituitary-Adrenal Axis their levels tend to be higher during the morning. Since the relationship between salivary and serum total cortisol concentration has been reported markedly non linear (Llena, 2006) due bounding to proteins in blood, it is important to determine cortisol concentrations in saliva (Hernández, 2012). However, only radiochemical and enzymatic (Elisa test) methods for quantification of cortisol in saliva have been reported (De Echeverri, 1995; Walsh, 2008). Therefore, the development of an inexpensive, faster and simpler analytical methodology to quantify

cortisol in saliva is seeked. Saliva is a fluid that is part of the oral cavity where dental therapies or treatments are performed, and the cortisol levels determination ensure adequate treatments to functionalize them with other compounds (antibiotics, nanoparticles, carbon nanotubes, analgesics, anti- inflammatory) to improve the therapy of each patient. Llena- Puy reported that saliva is a colorless, viscous liquid that moistens and moistens the oral cavity, it is a complex secretion which is mainly produced by the major salivary glands: parotid, submandibular and sublingual, and in a minor volume by the minor salivary glands: labial, buccal or vestibular, palatine and lingual (De Echeverri, 1995). It is a sterile liquid as soon as it leaves the salivary glands, and before to be mixed with the crevicular fluid, food remains, microorganisms, among others (Garde, 2005). Depending on the gland that produces and excretes saliva into the oral cavity, its consistency could be from very liquid to viscous. Saliva contains 99% water and 1% electrolytes (chlorine, calcium, sodium, potassium or bicarbonate); proteins such as enzymes (amylase, lysozyme or peroxidases), glycoproteins (mucins), salivary immunoglobulins, and hormones such as hydrocortisone that is released in periods of stress or in low concentrations of glucocorticoids in the blood (USP, 2007). The composition of saliva varies from site to site within the mouth of each individual, and changes according to the time of day (FDA, 2015), but it has been reported as a very stable biological fluid (FDA, 2000). Here, saliva can be used as a non-invasive method to monitor the plasma concentrations of drugs, hormones such as cortisol, or other substances. Cortisol quantification requires of a validated method.

The development of a validated analytical method is the process by which, through laboratory studies, the performed characteristics of the method meet the requirements for the intended analytical applications according to USP (Breaux, 2003). According to the Food and Drug Administration (FDA), a method validation is the process by which analytical procedures are shown to be suitable for the indicated use (Turpeinen, 2013). A validation process is the systematic study of linearity, precision, accuracy, reproducibility, repeatability, minimum quantifiable quantity (MQQ) and minimum detectable quantity (MDQ) (Farmacopea, 2017; Ranjita, 2005). Analytical methods using LC-MS/MS are chosen for measuring salivary cortisol. However, the lacking of a single and validated reference range, as well as their poor standardized assays, limit their use (Hernández, 2012). Thus, the aim of this work was to develop a validated spectrophotometric method considering the analytical procedures above described to quantify the free and active form of cortisol in saliva samples of young and older adults, in order to provide a low-cost method compared with the radiochemical and enzymatic methods previously reported (Corazza et al., 2007).

MATERIAL AND METHODS

Reagents and chemicals

Hydrocortisone Pharmaceutical Secondary Standard was purchased from Pfizer-Belgium NV (Belgium). Monobasic potassium phosphate, ethanol and trichloroacetic acid were obtained from Distribuidora Greep S.A. de C.V, México. All other chemicals were of analytical grade and the water was obtained from a Milli-Q System (Germany).

Apparatus

Ultracentrifuge (HERMLE Z 160 M). Thermo Scientific GENESYS 10S UV-VIS Spectrophotometer.

Procedure

Saliva samples from young people (n=35) and older adults (n=15) were used to validate the analytical method.

Biological sample

Salivary biological fluid (1.5 mL) was collected in Eppendorf tubes (Safe-lock) from subjects who were previously asked to fast, and whose ages ranged from 18 to 24 and 45 to 55 years. Tubes containing the samples were stored at 4 °C and used after 12 hours. Sampling was performed at the early hours of the morning, as cortisol levels have previously reported to be high (Corazza *et al*., 2007; Fastman *et al*, 2020). The study was approved by the Local Committee of the Autonomous University of Hidalgo State, México (Protocolo-001).

Extraction procedure

The nature and purpose of the study were explained to the volunteers and informed written consent was obtained from everyone. Saliva samples from subjects of two age groups, subjects whose ages ranged from 18 to 24 (25 women and 10 men) and ranged from 45 to 55 years old (8 women and 7 men) were used to validate the analytical method in order to determine cortisol concentrations. After storage at 4 ° C for 12 hours, saliva samples were centrifuged in an ultracentrifuge (HERMLE Z 160 M) at 11,000 rpm for 15 minutes. The centrifuged samples were placed in quartz cells to measure the absorbance in a UV-VIS spectrophotometer (ThermoScientific GENESYS 10S UV-VIS Spectrophotometer) to preliminary evaluate the presence of cortisol compared with other hormones secreted by saliva of the two different age groups (young and older adults). An extraction treatment using 5 mL of ethanol and 1 mL of trichloroacetic acid was also performed. The mixture was shacked for 5 min and the obtained solution was placed back in the 1 mL quartz cell to perform the cortisol extraction and measure the absorbance in a UV-VIS spectrophotometer at the maximum wavelength (280 nm).

Validation parameters of the analytical method

Specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), intra- and inter-day precision, and accuracy parameters were evaluated, as recommended by the guidelines of USP and FDA.

Specificity

The specificity of the method was evaluated for saliva samples to demonstrate that there was no interference in the quantification. Samples spiked with an ethanolic solution of the drug were used for this purpose. Hydrocortisone (cortisol) standard solutions were used to determine the maximum absorption wavelength of cortisol (n = 6) to further use it in the following procedures.

Linearity

To determine the linearity of the method, a six-fold calibration curve was prepared using a hydrocortisone standard solution (Pfizer-Belgium NV-Belgium) in concentration ranges from 2.5 to 25 μg/mL. Hydrocortisone standard solution of 0.25 mg/mL was prepared by dissolving 25 mg of hydrocortisone in 100 mL of ethanol. Magnetic stirring was applied for 5 min to ensure homogenization of the solution. Subsequently, six dilutions were prepared at 2.5, 5, 10, 15, 20 and 25 µg mL-1of hydrocortisone in saliva. The solutions were read in a UV-visible spectrophotometer at a wavelength of 280 nm. Quartz cells with a diameter of 1 cm were used. The absorbances were plotted versus each drug concentration. The linear coefficient of determination (R2) was calculated from linear regression analysis. According to the United States Pharmacopeia (USP 43) (Turpeinen, 2013; Stone, 2001), the R2 and the coefficient of variation must be higher than 0.98 and less than 3.0, respectively.

Accuracy

To evaluate the accuracy of the method, the analyte recovery from the biological samples was carried out. Six replicates were prepared at different concentrations: 2.5, 5, 10, 15, 20 and 25 μg/mL of hydrocortisone. Saliva samples were added with these known amounts of hydrocortisone ethanolic solutions to remain in contact with the drug for 30 min. After of 0.5 hours, samples were centrifuged in an ultracentrifuge (HERMLE Z 160 M) at 11,000 rpm for 15 minutes to measure the absorbance in a UV-VIS spectrophotometer (Thermo Scientific GENESYS 10S UV-VIS Spectrophotometer) at the same conditions described in the extraction section. The accuracy was expressed as the deviation percentage of the experimental drug concentration compared with that of the theoretical drug concentration. The standard deviation and the coefficient of variation were also determined for each concentration.

Precision

The precision of the analytical method was performed in terms of intra-day (repeatability) and inter-day and inter-analysts (reproducibility) for hydrocortisone samples in the biological media.

Repeatability

To evaluate this parameter, three replicates of six concentrations (2.5, 5, 10, 15, 20 and 25 µg mL–1) were evaluated by a single analyst. The samples were prepared at 2.5, 5, 10, 15, 20 and 25 µg mL–1 of hydrocortisone by dissolving 25 mg 25 of the drug in 100 mL containing ethanol. Subsequently, the samples were submitted to the extraction process and the drug was quantified on the same day, at the same conditions describe above.

Reproducibility

The reproducibility was carried out evaluating six replicates of four concentrations, prepared by three analysts in 3 different days. The samples were prepared as it is indicated in the repeatability procedure and the extraction process and the drug quantification were realized at the same conditions describe above, using the same equipment. The coefficient of variation was estimated and compared with that established by the USP (≤ 3%).

Limit of quantification (MQQ)

The quantification limit was determined by diluting the lowest concentration of hydrocortisone (2.5 μg/mL) of the calibration curve, in order to find the minimum quantifiable concentration detected in the equipment, with accuracy and precision.

Detection limit (MDQ)

In the same way, dilutions were prepared from the hydrocortisone concentration of 2.5 μg/mL. Subsequently, absorbance was measured of these dilutions, in order to find a precise minimum concentration of hydrocortisone at least 3-fold higher than that detected for the noise response.

Statistical analysis

The Cochran test was used to evaluate the accuracy data by comparing different concentrations and the proportions measured, considering a significance level at $p < 0.05$, when the data fit the Chi square distribution ($\chi 2$). **Student**-t test was used to determine significant differences between drug recovery averages. Fisher's tests and **Student**-t test were also applied to determine the significant differences and to evaluate the variation of the results from the experimental conditions of analysis.

RESULTS

The development of a low-cost analytical method to ensure the quantification of cortisol was addressed, complying with the specifications determined in the Pharmacopeia of the United Mexican States (USP). Linearity, precision, accuracy, reproducibility and repeatability results of the developed analytical method, are discussed in the following subsections.

Specificity

Figure 1 shows the absorption spectrum obtained to characterize and identify cortisol as a test of specificity. In this figure shows the spectra of the saliva sample before extraction and after extraction with ethanol and trichloroacetic acid. The figures show that there is no interference in the determination of cortisol in saliva samples where the wavelength of maximum absorbance was 280 nm.Figure 2 shows the maximum absorbance in the spectrum of hydrocortisone in water and the maximum absorption in saliva samples.

Linearity

To evaluate the linearity of the analytical method the calibration curve is displayed and the mathematical function and the graphical profile are shown in Figure 3. Table 1 shows the linearity parameters.

Accuracy and precision of the analytical method

For accuracy studies, the recovery percentages established with the use of a spectrophotometric method are in a range from 98 to 102%, in this case it was 100.05% and the value of the coefficient of variation was less than 3% (0.347). Table 2 shows the results obtained in the accuracy test of the analytical method. Table 3 shows the repeatability and precision results. Table 4 shows the reproducibility of cortisol.

DISCUSSION

Specificity

Evaluation of the specificity was performed firstly in samples of saliva prior to be processed with the extraction method, in order to determine the presence of the interferences to quantify cortisol hormone. Regarding the results obtained from the specificity procedure in saliva samples after the extraction process with ethanol and trichloroacetic acid, no interference was found in the determination of cortisol (Figure 1).

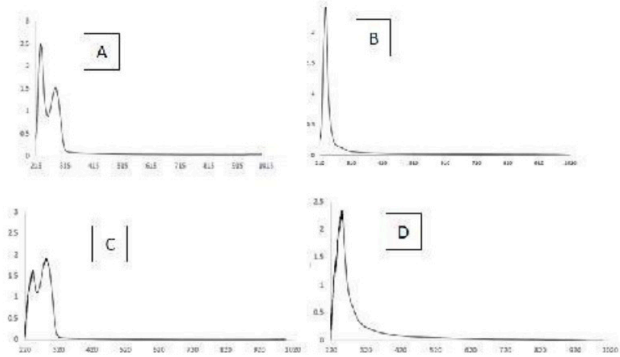

Fig. 1 Absorption spectrum of saliva samples before extraction with ethanol and trichloroacetic acid in young subjects: graphic A and after extraction see graphic C. In adults' subjects before extraction see graphic B and after extraction see graphic D, respectively

The wavelength of maximum absorbance for the drug was 280 nm in saliva. Moreover, the absorption spectrum obtained using standard hydrocortisone solutions was also specific to identify cortisol as it is shown in Figure 2. Thus, cortisol was able to be quantified from the components of the salivary matrices. In saliva samples, cortisol is not bound to proteins and its concentration is independent of salivary flow. Therefore, extractions with ethanol and trichloroacetic acid were carried out to degrade the different enzymes or proteins found in saliva samples, and, to leave free cortisol for its quantification. The absorption spectra obtained of hydrocortisonein distilled water and in saliva are shown in Figure 2.

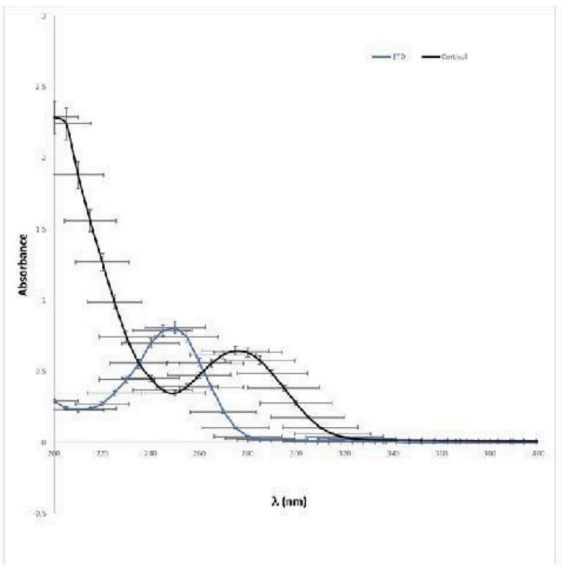

Fig. 2. Absorption spectrum of the hydrocortisone standard (0.809, λ = 250 nm) in distilled water and cortisol in saliva samples (0.631, λ = 280 nm), after extraction with ethanol and trichloroacetic acid.

Linearity

Analytical curves were prepared and analyzed in the concentration range of 2.5 to 25 μg/mL, and were used to evaluate the linearity of the analytical method. The regression equation and the graph obtained by plotting the absorbances versus each drug concentration are shown in Table 1 and Figure 3, respectively. Analyzing the linearity parameters obtained, such as the linear correlation coefficient of 0.9999 and the determination coefficient of 0.9997, it could be inferred that the response of the method was linearly proportional to the analyte concentration. Additionally, a calibration standard curve with a good quality was obtained due to the correlation coefficient value was higher than 0.99.

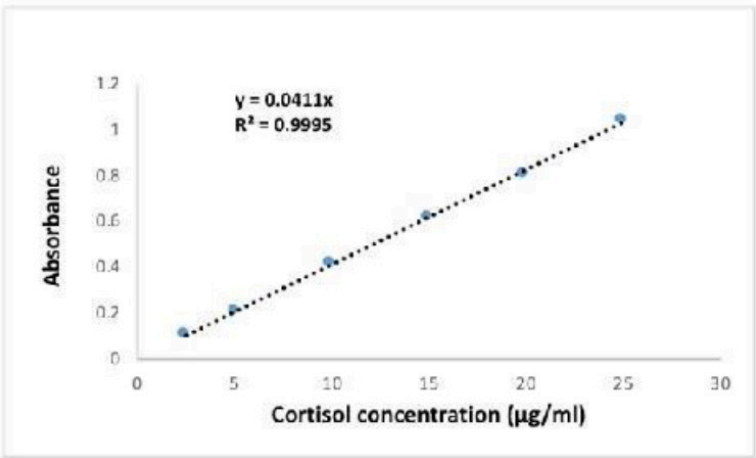

Fig. 3. Cortisol calibration curve, performed based on the analytical method developedat different concentrations in saliva1) 2.5 μg/ mL, 0.103; 2) 5 μg/mL, 0.208; 10 μg/mL, 0.413; 3) 15, 0.616; 4) 20, 0.809; 5) 25, 1.039, λmax = 280 nm

Parameters	*Values*
Maximum absorbance and maximum wavelength (nm)	0.809, 250
Linearity range (μg/mL)	2.5 – 25
Linear coefficient of determination (R^2)	0.9997
Regression equation	Y = 0.0411X+0.0001
Slope	0.0411
Intercept	0.0001
Detection limit (μg/mL)	0.019
Limit of quantification (μg/mL)	0.16

Table 1. Linearity parameters obtained from the Cortisol calibration curve.

Table 1 shows all the linearity parameters. The regression equation obtained corresponds to the ordinate at the origin with a slope that is the result of the interaction of the absorbance as a function of the cortisol concentration. The coefficient of variation obtained (1.03) met with the linearity parameters required by USP 31 (Breaux, 2003) and

USP 43of the linear responses (Stone, 2001), in whichthe variation coefficient must be less than 5%. Moreover, the standard deviation (Sb) values of the linear responses were less than 2%, indicating a good linearity of the analytical method developed, meeting the linearity criterion. A proportionality test of the ordinate at the origin, which must be zero for a linear function, was used to determine the systematic error. Results shown this error was small, since the Student-t test of 0.998 is less than the value of "t" (2.015) obtained from tables (*df* = 5 and *p* = 0.05). These results indicate the straight line passes through the origin and is statistically significant, confirming the proportionality condition. Additionally, the sensitivity of the analytical method was evaluated. The MQQ and MDQ values were 0.16 and 0.019 µg/mL, respectively. The concentration determined for the MQQ was very low, which is sensitive enough for quantification of low cortisol levels.

Accuracy and precision of the analytical method

For accuracy studies, the obtained results are shown in Table 2. According to the USP, the recovery percentages required when a spectrophotometric method is used must be in a range from 98 to 102%.

C(µg/mL)	*PR1(%)*	*PR2(%)*	*PR3(%)*	*PR4(%)*	*PR5(%)*	*PR6(%)*	*R(average)(%)*	*Sb*	*CV(%)*
2.5	100.97	99.04	98.06	100.99	100.98	100.97	100.17	1.29	1.29
5	100	99.52	101.45	98.57	100	99.52	99.84	0.94	0.95
10	100.73	99.04	101.21	99.04	99.76	100.24	100.003	0.89	0.89
15	100	99.03	101.48	99.84	99.03	100.82	100.03	0.98	0.98
20	100.50	100	99.02	100.99	98.89	100.50	99.98	0.86	0.86
25	99.81	100.39	99.04	100.39	99.90	100	99.92	0.50	0.50

Table 2. Cortisol Accuracy.

C= cortisol; PR=cortisol recovery percentage; PR average = average cortisol recovery percentage; Sb= standard deviation; CV= coefficient of variation.

C(µg/mL)	*PR1(%)*	*PR2(%)*	*PR3(%)*	*PR4(%)*	*PR5(%)*	*PR6(%)*	*PRaverage*	*Sb*	*CV (%)*
2.5	100	100.97	99.04	100	100	104.37	100.73	1.89	1.87
5	99.04	100.48	100.97	99.04	100.48	98.32	99.72	1.06	1.06
10	100	100	99.27	100.49	100.49	99.76	100.00	0.46	0.460
15	99.68	100.65	100.16	99.52	100.16	99.68	99.97	0.43	0.43
20	100.12	100.12	99.75	99.63	100.12	99.88	99.94	0.22	0.22
25	99.90	100.29	99.90	99.52	100.39	99.62	99.94	0.35	0.35

Table 3. Precision and repeatability of cortisol.

C= cortisol; PR=cortisol recovery percentage; PR average= average cortisol recovery percentage; Sb= standard deviation; CV= coefficient of variation.

Percentage recovered (%)	Analyst 1			Analyst 2			Analyst 3		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
	101.94	100	103.88	100.97	100.97	104.85	102.91	101.94	102.913
	100.48	99.52	98.56	100.48	99.04	99.038	97.59	99.04	100.96
	99.75	100.96	100.72	100.73	102.91	100.24	103.39	99.76	100.24
	99.67	98.70	100.49	100	100.81	99.84	99.51	101.46	99.84
	99.13	99.88	100.99	100.99	99.14	99.14	99.39	99.26	100.62
	100.57	99.23	99.32	99.04	98.65	98.65	101.64	101.44	100.58
Average	100.26	99.71	100.66	100.37	100.25	100.29	100.74	100.48	10.26
CV(%)	0.6	0.9	1.03	0.75	1.62	2.3	2.25	1.27	1.07

CV= coefficient of variation.

Table 4. Reproducibility of cortisol

In this study, the recovery percentage obtained was 100.05 %, and the variation coefficient was less than 3% (3.47). Statistically, it was verified using the Cochran test, showing a significant difference (P <0. 05) between each concentration (Q_{value} = 18.85 > Q_{tables} = 0.54, where k = 5 and nk = 25). The repeatability and precision procedures analyzed by 3 analysts for three days using the same equipment were performed in order to evaluate the variation intra and inter day. Regarding the intra and inter day reproducibility studies, 6 concentration levels were analyzed for each analyst, on the same day with three replicates, and the variation of each concentration level was determined by calculating their variation coefficients (Table 3). These coefficients of variation obtained were less than 2% per each concentration under study, indicating the analytical method to quantify cortisol is repeatable and reproducible (Table 3 and 4). The coefficients of variation obtained in this study are in agree with the established limits by the USP (To verify the precision of the analytical method, the Fischer test was performed, showing that there are no significant differences between the data obtained by each analyst or between each day (P > 0.05, $F_{calculated}$ = 0.985 < F_{tables} = 2,624). Moreover, no significant differences between treatments were found (tcalculated = 0.468 and ttables = 0 2.015, P > 0.05), verifying the method is precise to quantify cortisol.

Application of the validated analytical method to quantify cortisol from human saliva

Once the spectrophotometric method was developed and validated, it was used to quantify cortisol from saliva samples. The results obtained for average concentration in young subjects was of 52.58 ± 8.59 µg/dL and in older adults 38.83 ± 2.82 µg/dL using this method. This significant difference of cortisol between the two age groups, agrees with the previous reported in literature (Corazza, 2007), and is due to lower production of cortisol in older adults as the age increase. As mentioned above, cortisol is secreted in the hypothalamic-pituitary-adrenal axis and this compound influences changes in the circadian cycle in early hours or upon awakening in subjects of both sexes (Corazza, 2007). Thus,

the highest levels of cortisol in the saliva samples were analyzed in this study in the two age groups. It is important to mention that a variation of the cortisol concentration has been reported by using other analytical methods either from serum or saliva samples, including its determination in saliva from newborns (López, 2010; Fastman, 2020)[17-18]. In this case the free concentration of cortisol could be fully considered available, since cortisol diffuses through the intracellular route where it is taken by the glandular acinus and subsequently released by saliva (Fastman, 2020).

CONCLUSION

A validated spectrophotometric method was developed to quantify the free and active form of cortisol in saliva samples of young and older adults, in order to provide a low-cost method compared with the radiochemical and enzymatic methods previously reported. The analytical method is considered appropriate to quantify cortisol from saliva samples under the working conditions used in this study. This method complies with the specifications required by the Pharmacopeia of the United Mexican States (USP) in terms of the specificity, linearity, sensitivity, accuracy, and precision (reproducibility and repeatability).

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CONFLICT OF INTERESTS

There is no conflict of interest between authors.

GLOSSARY OF ABBREVIATIONS

FDA: Food Drugs Administration MQQ: minimum quantifiable quantity
MDQ: minimum detectable quantity
R_2: linear coefficient of determination
χ^2: Chi square distribution
F: fisher test
T: student test
Sb: standard deviation
df: degrees of freedom
Q: Cochran test
USP: United States Pharmacopeia

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