Determination of cortisol in human saliva using liquid chromatography–electrospray tandem mass spectrometry

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Abstract

The aim of this work was to develop a method for determination of cortisol in saliva by liquid chromatography–tandem mass spectrometry (LC–MS–MS). Saliva was sampled on Salivette tubes. These were centrifuged, deuterium-labeled cortisol was added as internal standard and the proteins precipitated by acetonitrile. The supernatant was evaporated, dissolved in methanol acidified with acetic acid and analyzed by LC–MS–MS. The with-in run precision, tested by pooling saliva samples from volunteers and then analyzing these in a single run, was found to be 7% at 0.7 μg l^{-1}. The between-run precision was tested by analysis of the same samples at different days and found to be 11% at 2.5 μg l^{-1}. The limit of quantification was 0.5 μg l^{-1}. The method was applied for analysis of saliva samples from three volunteers during their last week before vacation and the first and second week on vacation. In addition, the method was compared to analysis by an immunological method. The values from the immunological method were 2.7 times higher than the LC–MS–MS results.
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1. Introduction

Cortisol in serum and urine has long been used as a marker of adrenocortical function and urinary and serum cortisol concentrations may also serve as diagnostic tools for depressive disorders and chronic fatigue [1,2]. Moreover, cortisol may be used as a biomarker of stress [3–6]. However, for such mea-surements the sampling of blood may itself induce stress and the collection of urine represents a cumulative or averaged response.

Cortisol in saliva has been suggested as a stress biomarker for its direct measurements and stress-free collections. Furthermore, the sampling of saliva is easy to perform without medical supervision. Mea-surement of salivary cortisol has been found to be an excellent indicator of unbound concentrations of cortisol in serum [7] and a close correspondence in circadian fluctuations is found for salivary and plasma cortisol [8]. This makes salivary cortisol a candidate for monitoring of stress in large popula-

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tions of workers. On the other hand, a drawback with the use of saliva cortisol is the lower concentrations found as compared to serum and urine. The reference concentrations for cortisol in saliva are 1–8 μg l^{-1} in the morning and 0.1–1 μg l^{-1} in the evening, which is about two orders of magnitude lower than those in serum [9]. For this reason most reported methods for determination of saliva cortisol have been based on radioimmunoassays (RIA). These immunological methods are sensitive but have the drawback that there may be cross-reactivity with other steroids and these methods may therefore yield too high cortisol concentrations. A method using column-switching liquid chromatography (LC) with laser-induced fluorimetric detection for saliva cortisol has also been reported [10]. However, the interference from other compounds makes these analysis complicated and time consuming.

LC with tandem mass spectrometry (MS–MS) has recently started to be widely used in analysis of organic substances. The high sensitivity and selectivity of this method makes it possible to develop rapid analysis of very low concentrations even in complex biological materials. LC–MS–MS methods for analyses of cortisol in urine have previously been reported [11–13]. However, no method for analysis of salivary cortisol has been described. The aim of this work was to develop such a method. The method was validated, applied in healthy volunteers and compared to analysis using immunological methods.

2. Experimental

2.1. Apparatus

Analyses were performed with a Perkin-Elmer Series 200 liquid chromatography system with autosampler (Applied Biosystems, Norfolk, CT, USA), coupled to an API 3000 LC–MS–MS (Applied Biosystems/MDS-SCIEX, Toronto, Canada). The column was a Genesis C$_8$ (20×2.1 mm) with a particle size of 4 μm (Jones, Lakewood, CO, USA). For elution of the saliva from the wool swab and for sedimentation of the precipitate a Model 3E-1 centrifuge (Sigma, Deisenhofen, Germany) was used.

2.2. Chemicals

Cortisol (hydrocortison) was from National Institute of Standards and Technology (Gaithersburg, MD, USA). Tetra-deuterium-labeled cortisol was from Cambridge Isotope Laboratories (Andover, MA, USA). Methanol and acetonitrile were from LabScan (Dublin, Ireland). Glacial acetic acid was from Merck (Darmstadt, Germany). Water was purified using a Maxima HPLC Mark II system (ELGA, Bucks, UK).

2.3. Sampling and storage

Saliva samples were collected in Salivette tubes containing a polyester wool swab (Sarstedt, Nümbrecht, Germany). After sampling the samples were stored at −20 °C until analysis.

2.4. Preparation of standards

A stock solution of cortisol was prepared by dissolving 20 mg cortisol in 10 ml of methanol. This solution was further diluted in methanol:water (50:50) containing 0.5% acetic acid to desired concentrations (0.8–80 μg l^{-1}). Standards were prepared by addition of 100 μl of these solutions to 250 μl water.

2.5. Work-up procedure

The Salivette tubes were thawed and centrifuged at 2700 *g* for 15 min to elute the saliva. Saliva (250 μl) were added with 0.6 ng deuterium-labeled cortisol in 100 μl methanol:water (50:50) aliquots containing 0.5% acetic acid. To precipitate proteins 500 μl acetonitrile, acidified with 0.5% acetic acid, was added. The samples were then mixed and conditioned at room temperature for 10 min after which the samples were centrifuged at 2700 *g* for 15 min. The supernatants were evaporated in a nitrogen flow and dissolved in 100 μl methanol containing 0.5% acetic acid. The samples were stored at 4 °C until analysis.

2.6. LC–MS–MS analysis

Aliquots of 5 μl were injected by the auto-sampler onto the column. The needle was rinsed twice before and twice after the injection by 250 μl methanol. Mobile phase was a water–methanol gradient containing 0.5% acetic acid. The initial mobile flow was 50% methanol. A linear gradient to 100% methanol was applied in 3 min whereafter the column was conditioned at 50% methanol for 2 min. The mobile flow-rate was 0.2 ml/min. The turbo ion spray interface was set to 370 °C, the ion spray voltage to 4500 volts and the declustering potential was 50 volts. For cortisol, analysis after collision induced fragmentation of the precursor ion at m/z 363.0 was performed at m/z 309.0 with a collision energy (CE) of 26 volts. For the tetra-deuterium-labeled cortisol the precursor ion at m/z 367.0 was fragmented to m/z 313.0 with CE 26 volts. Another fragmentation of cortisol was m/z 363.0/120.8 (CE 44 volts) and for deuterium-labeled cortisol m/z 367/120.8 (CE 44 volts) but these mass fragments were not used because of co-eluting interfering compounds. The peak area ratios between the analytes and the internal standards were used for quantification.

2.7. Radioimmunoassay (RIA) for cortisol

A competitive RIA (Spectria Cortisol Coated Tube RIA) purchased from Orion Diagnostica, Espoo, Finland was used for the determination of cortisol in saliva. The assay was designed for quantitative in vitro measurement of cortisol in serum, plasma, urine, and saliva. The analysis was carried out according to the manufacturers specifications. The sample volume was 150 μl, the range of the standard solution prepared was 0.36–36.2 μg l^{-1}, and incubation time was 30 min at 37 °C. The sensitivity was given by the manufacturer as twice the standard deviation of the zero-binding value in saliva to be 0.3 μg l^{-1}, bias to be 110% (103–115%), intra-assay variation to be 5.4% and inter-assay variation to be 7.3%. The cross-reactivity of the antiserum used were tested for e.g., 5α-dihydrocortisol (84.3%), 21-desoxycortisol (78.8%), prednisolone (45.3%), 5β-dihydrocortisol (11.9), and 6α-methylprednisolon (11.0%). Cross-reactivity to corticosterone and cor-

tisone was <0.2%. A 1470 Wizard gamma counter (Wallac, Turku, Finland) was used for measurement of radioactivity.

2.8. Statistics

For comparison of the LC–MS–MS method and the RIA a functional model $E(Y_i) = b*E(X_i) + a$, where b denotes the slope and a denotes the intercept, was estimated and the approximate standard deviations (SD) of the estimates of a and b were calculated [14]. The analysis allowed for adjustment of differences in variation of the two different analytical methods by use of the factor $\lambda = \sigma_y^2/\sigma_x^2$.

3. Results and discussion

A fast and simple work-up procedure was preferred. However, there was a need both to eliminate proteins and to concentrate the saliva. Thus, the saliva was added with twice its volume with acetonitrile. This procedure has been shown to eliminate 99.7% of the proteins in plasma [15]. Furthermore, the supernatant was evaporated into dryness and re-dissolved in mobile phase giving a 2.5-fold concentration of the cortisol.

LC–MS–MS equipment are expensive instruments and high through-put analyses are important for economical reasons. Thus, we tried a short 2 cm with a C_8 stationary phase. Moreover we used a fast gradient of 50 to 100% methanol in 3 min and a short re-conditioning of the column at 2 min. At these conditions cortisol eluted after approximately 2 min in a sharp symmetrical peak. A chromatogram of saliva from a healthy volunteer containing 0.5 μg l^{-1} is shown in Fig. 1.

Positive ion single MS mass spectra of cortisol and tetra-deuterium-labeled cortisol have been reported previously using atmospheric pressure chemical ionisation [12,13]. In addition, a mass spectrum from a collision induced fragmentation of the cortisol molecular ion (M+H) have been reported showing 121.4 as a major fragment [12]. In this study, collision-induced fragmentation of M+H ($m/z =$ 363.0) at 363.0 gave two major fragments at m/z 309.0 and 120.8. The corresponding fragments for
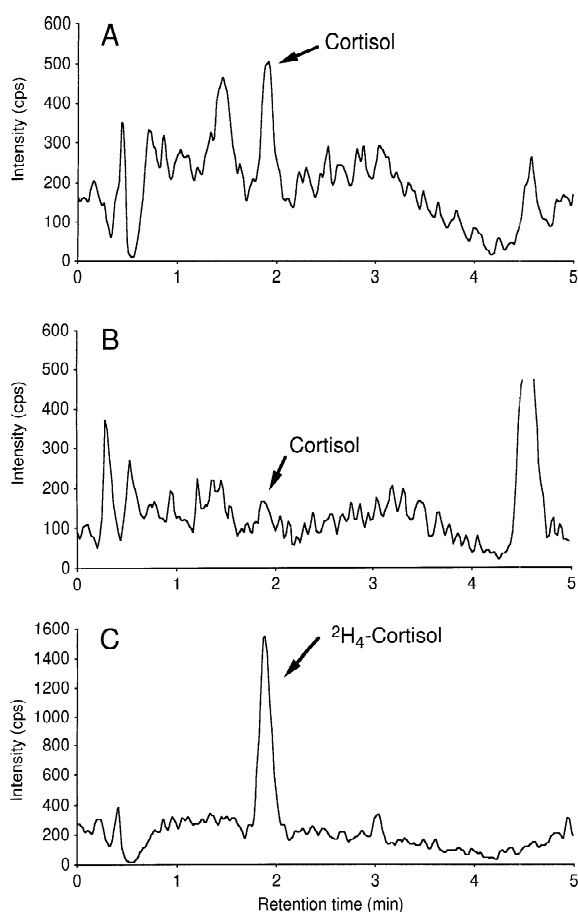
Fig. 1. Chromatograms showing a saliva sample with (A) a cortisol concentration of 0.5 μg l^{-1}; (B) a saliva below the detection limit; and (C) the deuterium-labeled internal standard.

the deuterium-labeled internal standard was m/z 313.0 and 120.8. Both fragments were evaluated for use in the analysis of cortisol. Initial tests showed that the 120.8 fragment had a slightly higher sensitivity but co-elution of a interfering substance made quantification impossible at this fragment. Not even the use of a longer column (Genesis C_{18}, 50×2.1 mm) gave separation of the peaks and longer columns were not tried since fast separations were preferred. Thus, m/z 363.0/309.0 were chosen for the determinations of cortisol and the corresponding fragment at m/z 367.0/313.0 were used for the internal standard.

For practical reasons standards were prepared in pure water. A test was performed to ensure that water standards were applicable for the determinations. Standards up to 30 μg l^{-1} cortisol were prepared in water and in saliva from a healthy volunteer. The experiment was repeated twice. The original content of cortisol in the saliva was determined by the standard addition method to 4.0 μg l^{-1}. Comparing the slopes of the regression lines showed that standards in saliva gave 93% of that of water in both experiments. Thus, standards in water seem to give almost similar determinations as compared to saliva standards. Correlation coefficients of the regression line of the standard curve was typically >0.999. The curves were linear over the whole range.

The recovery of cortisol in the samples was determined to 64% by comparison of peak areas of spiked saliva samples with pure standards in methanol. The loss of cortisol probably originates from the precipitation step where all of the sample can not be transferred to the new tubes. However, this loss is probably not of importance for the accuracy of the analysis since deuterium-labeled cortisol was used as internal standard.

The within-day precision was tested using saliva samples pooled from healthy volunteers. The pooled saliva samples were worked-up and analyzed according to the LC–MS–MS method in a single run. The coefficient of variation (C.V.) was found to be 7% at 0.7 μg l^{-1} ($n=13$). The between-day precision was studied by analysis of real saliva samples analyzed in duplicate on different days. The C.V. for 34 duplicate samples at 2.5 μg l^{-1} was found to be 11%. The limit of quantification, defined as the concentration giving a between-day precision at 25%, was 0.5 μg l^{-1}.

The detection limit, defined at three times the amplitude of the noise in saliva samples, was 0.2 μg l^{-1}. Thus, concentrations of cortisol in saliva sampled from normal people in the evening are close or even below the detection limit. However, one strategy for the use of cortisol as a stress biomarker is to collect several saliva samples in the morning to establish the awakening response and subsequently the decline during the day to establish the variability which is related to development of metabolic syndrome [3,16]. As it still is uncertain which

cortisol measure that is the best indicator of chronic stress we suggest evaluating the area below the cortisol concentrations as a biomarker. Thus, it is the highest cortisol concentrations that strongest influence this area.

The method was applied on saliva samples from three healthy volunteers on the Wednesday before they went on vacation and the first and second Wednesdays during vacation. Four samples per volunteers were collected each day, immediately when wakening up, after 45 min, after 8 h and at 20.00 in the evening. The results are shown in Fig. 2. Thus, the method is applicable for analysis of samples from the normal population. There was no indication of lower concentrations of cortisol during the vacation.

A comparison was performed between the LC–MS–MS method described here and the RIA method (Fig. 3). The RIA method gave cortisol concentrations, which were 2.7 times [95% confidence interval (CI) 2.1–3.5] higher than the LC–MS–MS method, when including only samples above the detection limit. For some samples the RIA method gave much higher concentrations than the method presented in this study. The reason for this discrepancy is not clear but cross-reactivity with other steroids in the RIA method may be an explanation.

The method has also been applied in an interlaboratory comparison program together with eight other laboratories [17]. The method was the only one
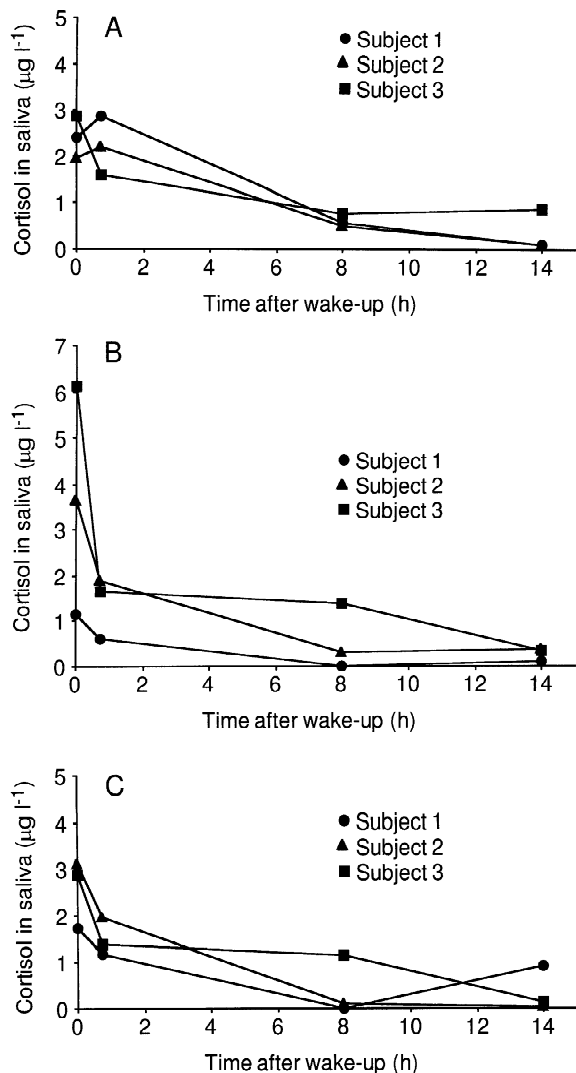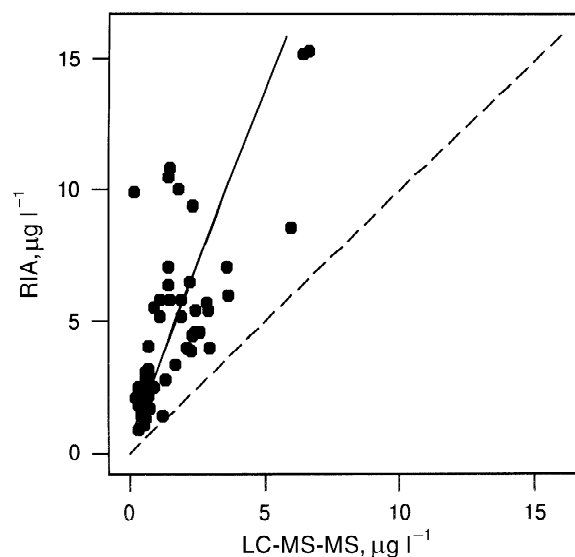

Fig. 2. Cortisol concentrations in healthy volunteers determined by LC–MS–MS on (A) the Wednesday before vacation, (B) the Wednesday the first week on vacation and (C) the Wednesday the second week on vacation. The samples were collected immediately on wakening up, after 45 min, after 8 h and at 20.00 in the evening.

Fig. 3. Comparison between cortisol in saliva determined by the method described here (LC–MS–MS method; x-axis) and the RIA method (y-axis). The equation from the functional model is $y = 2.66x + 0.50$, $n = 57$. In addition, $x = y$ is included as a broken line.

Table 1
Spiked and estimated concentrations for salivary cortisol in an inter-laboratory comparison using LC–MS–MS and RIA determinations

Spiked concentration ($\mu g\ l^{-1}$)	Estimated concentration by LC–MS–MS ($\mu g\ l^{-1}$)	Estimated mean concentration by all laboratories ($\mu g\ l^{-1}$)
0.0	1.1	2.5
3.5	4.4	6.6
7.5	7.2	11.0
14.9	13.6	18.6
24.8	24.9	29.4

using the LC–MS–MS technique; all other laboratories used immunologically based methods. Five samples were analyzed. They were prepared from a pool of natural saliva samples, which were spiked with a certified reference cortisol from National Institute of Standards and Technology (standard reference material 921). The spiked and estimated concentrations are shown in Table 1. The recovery of the spiked material by the LC–MS–MS method was estimated to be 91% (C.I. 76–106%). The concentration of cortisol in the pooled saliva without spiking was 1.1 $\mu g\ l^{-1}$ by the LC–MS–MS method, whereas the mean result of all laboratories was 2.3 times higher. This corresponds well with the above presented discrepancy between LC–MS–MS and the RIA method. Thus, the differences between the methods appear to originate from the determinations of the natural cortisol concentrations in the pooled saliva rather than from the recovery of spiked cortisol.

Acknowledgements

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