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Development and evaluation of a liquid chromatography tandem mass spectrometry method for simultaneous determination of salivary melatonin, cortisol and testosterone

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ABSTRACT

Circadian disruption can have several possible health consequences, but is not well studied. In order to measure circadian disruption, in relation to shift or night work, we developed a simple and sensitive method for the simultaneous determination of melatonin, cortisol and testosterone in human saliva. We used liquid–liquid extraction (LLE) followed by liquid chromatography coupled to electrospray tandem mass spectrometry (LC–ESI–MS/MS) recorded in positive ion mode. Saliva samples were collected by spitting directly into tubes and 250 μL were used for analysis. The limits of detection were 4.1 pmol/L, 0.27 nmol/L and 10.8 pmol/L for melatonin, cortisol, and testosterone, respectively. The developed method was sensitive enough to measure circadian rhythms of all 3 hormones in a pilot study among four healthy volunteers. It can therefor be used to study the impact of night work and working in artificial light on the workers circadian rhythms. To our knowledge this is the first LC–ESI–MS/MS method for simultaneous determination of salivary melatonin, cortisol and testosterone.

1. Introduction

Circadian disruption can have many possible health consequences [1,2]. Laboratory studies of humans have shown that lack of synchronisation of circadian rhythms, measured by sleep and cortisol release, may cause a pre-diabetic change [3]. The internal balance of testosterone and cortisol has an effect on the risk of developing cardiovascular disease and type 2 diabetes [4]. In addition, the International Agency for Research on Cancer has classified shift work that involves circadian disruption as a probable human carcinogen [5]. Lack of coordination between circadian rhythms develops because different biological rhythms change with different speed [6]. Melatonin, cortisol and testosterone all have diurnal changes in concentrations [7–10]. Thus, the combined changes in the concentrations of all three hormones can be used as a robust marker of circadian disruption. Touitou et al. used salivary melatonin and cortisol to measure circadian profiles in prepubertal boys, however they were not able to measure testosterone due to lack of sensitivity in the applied assay [10]. Circadian disruption may be specifically interesting in relation to night work or work without daylight. Phase shifts in the human circadian system in both cortisol and melatonin can be affected by daylight and has been shown to adapt to the length and intensity of light exposure [11]. Further, cortisol, and testosterone in saliva have been used to study the circadian disruption among construction workers with 12-h workdays and extended workweeks [12].

Melatonin is synthesized and secreted primarily by the pineal gland [13] and functions as a circadian pacemaker that synchronizes the internal hormonal environment to the light–dark cycle of the external environment and has importance for season variability [14]. Melatonin has been shown to be a good biomarker of circadian dysregulation [8] and therefore melatonin is often used in studies to evaluate the level of circadian disruption as a result of night or shift work. Cortisol is produced in the adrenal gland and is the principal marker for the activation of the hypothalamus–pituitary–adrenal (HPA) axis [15]. The HPA axis plays a central role in homeostatic processes and it is commonly thought to reflect attempts to adjust to daily pressures and joys [16] and is one of the major physiological stress responses in the body. Cortisol is used as a biomarker in many studies of physiological stress [17–19]. Cortisol has a characteristic and stabile diurnal rhythm with a rise 30 min after awakening [14] and is a good diurnal rhythm marker [20]. Harris et al. used

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cortisol as a marker of diurnal rhythm in a study of offshore shift workers [7]. Testosterone is an anabolic steroid produced in the testes in men and in the ovaries in females and in the adrenal glands in both sexes. There is a slight diurnal variation in both sexes and testosterone is highest in the morning after a good nights sleep [9]. Testosterone has been shown to be a good biomarker for adverse psychosocial working environment for both men and women [21].

Melatonin, cortisol and testosterone can be measured in blood [18,22,23], urine [24–26] and saliva [27,28]. However saliva sampling is preferred since it has several advantages: It is noninvasive, painless and easy to perform [29]. Thereby multiple samples can easily be taken during the day in field studies where the participants in a study can be instructed in the sampling procedure at home and forward the samples by mail [30]. Several studies have shown that melatonin, cortisol and testosterone all have good correlations between saliva and serum levels of hormones [8,31,32].

Salivary hormones have, traditionally, been measured by immunochemistry based methods such as radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA) [33–36]. Examples of LODs using theses methods are: 1.29 pmol/L for melatonin (ELISA kit from DRG International Inc., NJ, USA), 0.8 nmol/L for cortisol (RIA kit from Origon Diagnostica, Espoo Finland) and 19.66 pmol/L for testosterone (ELISA kit from Abnova, Heidelberg, Germany). Although immunochemistry based methods are highly sensitive and also simple to use, they suffer inherently from a potential risk of cross-reactivity to structurally similar compounds [37]. In addition, RIA has been shown to be sensitive to both the collection procedure and the type of antibody used making it difficult to compare results obtained on different RIA kits [38].

When studying the working environment we primarily analyze samples from healthy adults. Thus, the variations in the concentrations of the hormones of interest are relatively small [39]. Hence it is crucial to use a precise and sensitive method making liquid chromatography tandem mass spectrometry the method of choice [40]. In addition, the time and resources spent on analyzing the hormones can be reduced dramatically by analyzing them in the same assay.

Several procedures for quantification of salivary hormones by LC–MS/MS have been reported previously [27,28,41–46]. Turpeinen et al. proposed a simple liquid–liquid extraction (LLE) procedure where 0.1 mL saliva were extracted with dichloromethane and got a LOD for cortisol of 0.07 nmol/L [44]. Kataoka et al. got a slightly better LOD for cortisol (0.05 nmol/L) [46] by use of an online solid phase micro extraction procedure. Motoymama et al. [47] used column-switching semi-micro column liquid chromatographic system with three columns to attain large volume injection and online enrichment for the measurement of melatonin. 400 μL saliva was injected and the estimated the LOD was 10.7 pmol/L [47]. In another study, Eriksson et al. used solid phase extraction (SPE) on 1 mL saliva sample followed by evaporation to dryness and reconstitution in methanol–water yielding an estimated LOD for melatonin of 4.5 pmol/L [28]. Matusi et al. developed a method based on LLE followed by evaporation to dryness and reconstitution in acetonitrile for the simultaneous determination of salivary cortisol and testosterone obtaining LODs of 0.03 nmol/L and 17 pmol/L, respectively [27]. There are other examples of studies where two/several hormones are detected simultaneously in human saliva [27,43,48], however no procedure has been reported for the simultaneous determination of melatonin, cortisol and testosterone. Thus, the aim of the present study was to develop and evaluate a simple and robust method that facilitates simultaneous determination of all three hormones.

2. Experimental

2.1. Chemicals

Melatonin (>97%), cortisol (>98%), testosterone (>99%), D-3-testosterone (>99%), D-4-cortisol (>99%) methanol (MeOH), ethyl acetate, formic acid and ammonium acetate were obtained from Sigma–Aldrich (St. Louis, MO). All solvents and additives were MS grade. D-4-melatonin (98%) was obtained from Qmx Laboratories (Essex, UK).

2.2. Preparation of standard solutions

Standard solutions were prepared from stock solutions in MeOH. For standard curves seven concentration levels were prepared for each hormone in 10% MeOH. Melatonin: 15.0–579.4 pmol/L, cortisol 0.5–90 nmol/L, and testosterone: 15.6–622.8 pmol/L. Internal standards were added to a final concentration of 1073 pmol/L, 25 nmol/L and 865 pmol/L for D-4-melatonin, D-4-cortisol and D-3-testosterone, respectively.

2.3. Sampling and sample preparation

Samples for the method evaluation consisted of female saliva collected in the late afternoon by spitting directly into conical polypropylene sample tubes (75 mm × 16.8 mm, Sarstedt, Nümbrecht, Germany). Samples for analysis of circadian rhythms were collected from 4 healthy volunteers; 3 women and 1 man, with ages ranging from 30 years to 55 years. The volunteers were instructed to spit directly into the tubes 6 times: at awakening, 30 min after awakening, at 12.00 h, at 16.00 h, at 20.00 h and right before bedtime. The samples were collected on a workday in the spring 2011. All saliva samples were stored at −20 °C until use. At the day of analysis, the samples were left to thaw at room temperature for approximately 45 min and were then centrifuged at 3500 g for 10 min. LLE was carried out by adding 63 μL internal standard and 1.0 mL ethyl acetate to 250 μL of a saliva sample in a 15 mL polypropylene tube. The resulting mixture was subsequently shaken for 45 min on a shaking table. The samples were centrifuged for 5 min at 3500 g and then placed in a freezer (−20 °C) for approximately 30 min. The ethyl acetate layers was poured off and evaporated to dryness under nitrogen. The residues were redissolved in 250 μL 10% MeOH.

The following biological relevant concentration ranges were used for the three hormones: melatonin: 15.0–579.4 pmol/L, cortisol: 0.5–90.0 nmol/L, and testosterone: 15.6–622.8 pmol/L.

2.4. Chromatographic conditions

A volume of 25 μL was injected into an Agilent 1200 HPLC (Agilent technologies, Santa Clara, CA, USA) equipped with a C18 2.1 mm × 50 mm 2.6 μm Kinetex column and a Krud-katcher ultra filter (Phenomenex, Torrance, CA). The mobile phase consisted of a 2 mM aquatic solution of ammonium acetate with 0.1% (v/v) formic acid (A) and MeOH with 2 mM ammonium acetate and 0.1% (v/v) formic acid (B). A linear gradient was run over 3 min from 10% to 100% B and maintained at 100% MeOH for 2.5 min, followed by 1 min of equilibration at 10% MeOH resulting in a total run time of 6.5 min. The flow rate was 0.5 mL/min and the temperature of the auto sampler and column oven was 8 °C and 40 °C, respectively.

2.5. Mass spectrometric detection

The mass spectrometer, an Agilent 6460 QQQ (Agilent technologies, Santa Clara, CA) equipped with a jet stream ESI ion source, was operated in the positive ion mode. The flow and temperature of the

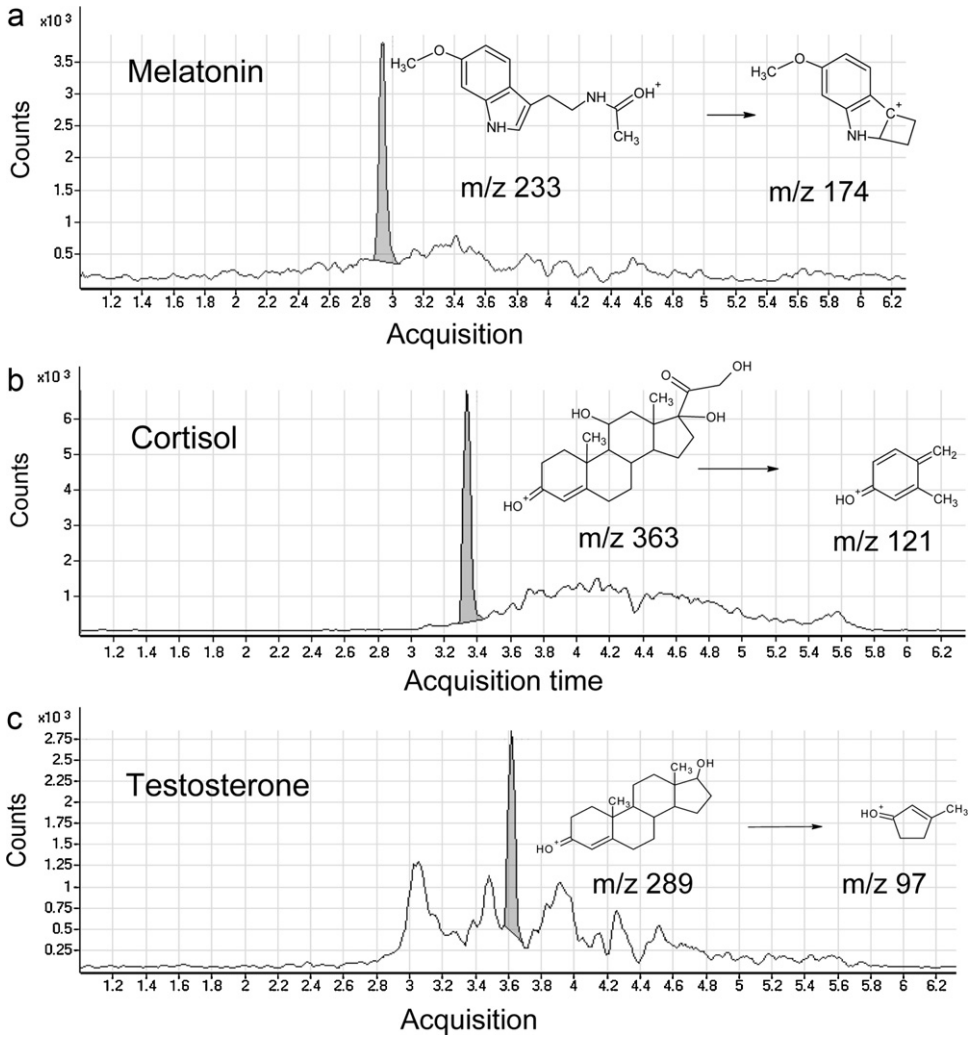

Fig. 1. Chromatogram of melatonin (25 pmol/L), cortisol (1 nmol/L) and testosterone (25 pmol/L) in saliva.

dry and sheath gases were: 5 L/min, 350 °C, 11 L/min and 400 °C, respectively. The nebulizer gas pressure was 50 psi and the capillary voltage was 4 kV. The quantification was achieved by using the mass spectrometer in multiple reaction monitoring (MRM) mode. A single precursor ion-product ion transition was measured for each hormone and its internal standard. The transitions were: m/z 233.2 → m/z 174.1 for melatonin; m/z 237.2 → m/z 178.1 for D-4-melatonin; m/z 363.2 → m/z 121.1 for cortisol; m/z 367.2 → m/z 121.2 for D-4-cortisol m/z 289.2 → m/z 97 for testosterone; m/z 291.2 → m/z 97 for D-3-testsoterone, see Fig. 1. The collision energies were 15, 18 and 20 eV for melatonin/D-4-melatonin, cortisol/D-4-cortisol and testosterone/D-3-testsoterone, respectively.

2.6. Method validation

The method evaluation was performed using the program AMI – Quality Assessment Scheme (AMIQAS). This program is designed in accordance with the recommendation of the ISO 5725 guideline and other official recommendations [49]. Limit of detection (LOD) was determined according to International Union of Pure and Applied Chemistry (IUPAC) as 3× standard deviation of a low sample injected 20 times [50]. For the study of intra- and inter-day precision saliva samples were prepared in two levels for each hormone, for melatonin: 76 pmol/L and 380 pmol/L, for cortisol: 13 nmol/L and 65 nmol/L and for testosterone: 82 pmol/L and 409 pmol/L. Samples were measured 10 times within the same run and in 10 consecutive runs.

2.7. Storage and stability

Saliva samples were spiked with all three hormones at seven levels in the following ranges: melatonin: 15.0–579.4 pmol/L, cortisol: 0.5–90.0 nmol/L, and testosterone: 15.6–622.8 pmol/L. For long term stability samples were measured once after production and kept at −20 °C until measured again after three months. For short term stability samples were left at room temperature and measured on day 1, 3 and 7.

3. Results and discussion

3.1. Sample preparation

We established a simple procedure by freezing the sample after LLE, making it possible to simply pour of the ethyl acetate layer as done by Matsui at al [27]. Use of LLE for sample purification yielded optimum speed and efficiency of the analysis and reduced the number of steps involved in sample preparation and thereby the number of possible sources of error. We found that reconstitution in 10% MeOH gave the best chromatographic separation. This procedure worked well for all three hormones and minimized our problems with ion suppression. In field studies it is often a problem

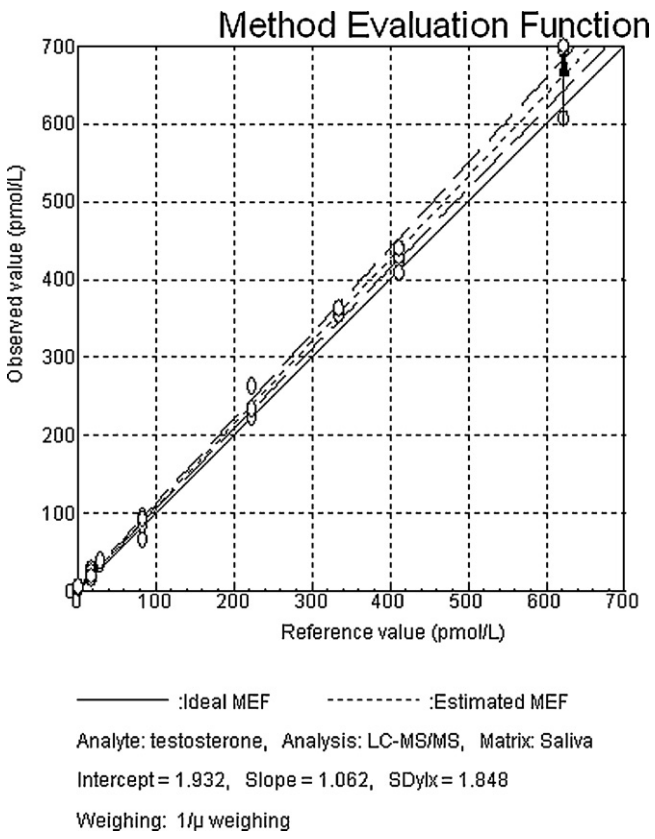

Fig. 2. Method evaluation function for testosterone in saliva. The measured concentration of 28 spiked saliva samples plotted against the corresponding true values.

to get enough saliva for analysis [29], therefore it is crucial to limit the amount of saliva needed for the analysis. Another advantage lies in the simplicity of sample preparation; LLE does not require a lot of expensive equipment and is easily performed.

Our initial goal was to inject saliva directly or to do a simple on line clean up as done by Perogamvros et al. [43]. However, we experienced a large amount of ion suppression as described by Annesley [51]. We tested different solid phase extraction (SPE) procedures, but did not get optimal results (data not shown).

3.2. Chromatography and method evaluation

Fig. 1 shows representative chromatograms for all three hormones. We achieved full separation at baseline of all three compounds. Fig. 2 shows the method evaluation function of testosterone as an example and Table 1 presents the method evaluation for all three hormones. The linearity of the method evaluation functions (MEFs) for all 3 hormones was tested using a pure lack of fit test, which was not significant at the 5% level, thus demonstrating linearity for all three MEFs. LOD were estimated using the definition by International Union of Pure and Applied Chemistry (IUPAC) [50] i.e., calculated as 3× standard deviation of a low sample injected 20 times, this can result in higher, but more realistic LODs [50].

Table 1
Method evaluation parameters for melatonin, cortisol and testosterone in saliva.

	Melatonin	Cortisol	Testosterone
Intercept	2.44 pmol/L	0.35 nmol/L	1.93 pmol/L
Slope	1.14	1.32	1.06
SD	1.45	1.66	1.85
LOD	4.11 pmol/L	0.27 nmol/L	10.81 pmol/L

The accuracy of the method in the full concentration range was evaluated in method evaluation as the recovery after adjusting for endogenous hormone content. We achieved recoveries of 114%, 132% and 106% for melatonin, cortisol and testosterone, respectively, equivalent to an accuracy that is 14%, 32% and 6% to high for melatonin, cortisol and testosterone, respectively. The high recovery could stem from multiple sources. One possible source of error could be that the internal standards is not as efficiently extracted or ionized as the spiked hormone. Another possibility is that the target drugs and their isotope labelled standard are suppressing each other during ionization [52]. Matrix effects in biological samples are many can be diverse in their effect on ionization ranging from almost full ion suppression to enhancement of ionization [51].

3.2.1. Melatonin

Fig. 1a shows a representative chromatogram of a saliva sample spiked with 25 pmol/L melatonin. The slope of the MEF for melatonin was 1.14 with a 95% confidence interval of [1.10; 1.17]. The intercept was 2.44 pmol/L saliva and significantly different from zero. The intercept of the MEF was significantly different from zero after adjusting for the endogenous amount of melatonin, indicating that we have a systematic error in our method. Intra-day precision was 8.3% for a concentration of 76 pmol/L and 5.1% for a concentration of 380 pmol/L. Inter-day precision was 17.5% for a concentration of 76 pmol/L and 11.6% for a concentration of 380 pmol/L. We found the LOD to be 4.11 pmol/L for melatonin and our method is thus slightly more sensitive than those reported by Eriksson et al. (LOD of 4.51 pmol/L) and Motoyama et al. (LOD of 10.73 pmol/L) [28,47]. Further, the amount of saliva needed for the analysis in our method was only 250 μL saliva and as little as 100 μL was adequate for analysis, since we do not need an up-concentration step. In both previously published methods on melatonin 1 mL of saliva was used [28,47].

3.2.2. Cortisol

Fig. 1b shows a representative chromatogram of a saliva sample spiked with 4.3 nmol/L cortisol. The slope of the MEF for cortisol was 1.32 with a 95% confidence interval of [1.23; 1.34]. The intercept was 0.35 nmol/L saliva and not significantly different from zero after adjusting for endogenous cortisol, indicating no systematic error in the analysis. Intra-day precision was 8.5% for a concentration of 13 nmol/L and 7.8% for a concentration of 65 nmol/L. Inter-day precision was 14.3% for a concentration of 13 nmol/L and 13.4% for a concentration of 65 nmol/L. The LOD for cortisol was found to be 0.27 nmol/L. Several other HPLC–MS–MS analysis of cortisol in human saliva exists [27,41–43]. Since we have optimized our method for the simultaneous analysis of cortisol, melatonin and testosterone we have not been able to achieve a sample preparation as simple as done by Perogamvros [43]. However, we still obtain a LOD within the relevant biological range. Perogamvros et al. [43] did a simple online clean up and archived a LOD of 0.39 nmol/L and reported no ion suppression. The method developed by Matsui et al. can also be used to simultaneous determine testosterone and cortisol in saliva [27], but it requires a 10 times up-concentration and melatonin is not included in this method.

3.2.3. Testosterone

Fig. 1c shows a representative chromatogram of a saliva sample spiked with 25 pmol/L testosterone. The slope of the MEF for testosterone was 1.06 with a 95% confidence interval of [1.03; 1.10]. The intercept was 1.93 pmol/L and significantly different from zero after adjusting for endogenous testosterone, indicating that we have a systematic error in our analysis of testosterone. Intra-day precision was 5.3% for a concentration of 82 pmol/L and 7.5% for a concentration 409 pmol/L. Inter-day precision was 15.2% for a concentration of 82 pmol/L and 9.0% for a concentration of 409 pmol/L. The

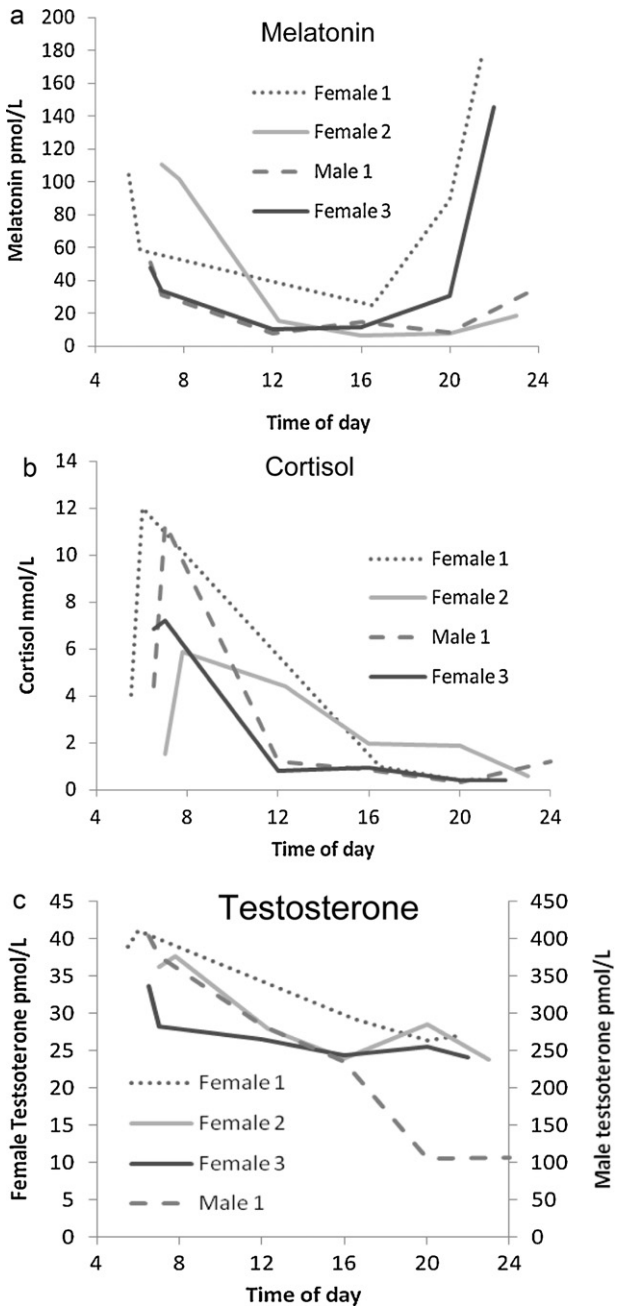

Fig. 3. Measurements of melatonin, cortisol and testosterone in saliva from 4 healthy adult volunteers. Saliva samples were taken at awakening, 30 min after awakening, at 12.00 h, at 16.00 h, at 20.00 h and just before bedtime.

detection limit of the developed method was 10.81 pmol/L and well in the biological relevant range of testosterone in saliva for both men and women. Further the method had a similar or better LOD than the 2 other published methods [27,48]. Matsui et al. used a similar extraction technique, but required four times more saliva (1 mL) to obtain a LOD of 17 pmol/L [27]. Shibayama et al. used SPE extraction and subsequent derivatization with 2-hydrazino-1-methylpyridine to get a LOD of 34 pmol/L using 500 μL saliva [48].

3.3. Storage and stability

For long term stability spiked saliva samples of all three hormones at seven levels (melatonin: 15.0–579.4 pmol/L, cortisol: 0.5–90.0 nmol/L, and testosterone: 15.6–622.8 pmol/L) were measured once after production and keep at −20 °C until they were measured again after three months. Samples were found to stable after three months at −20 °C, with a mean relative SD of 11.1%, 9.6% and 13.1% for melatonin, cortisol and testosterone, respectively. This is in line with previous studies showing that cortisol was stabile in saliva at −20 °C for up to one year [30] and that testosterone was stabile in saliva at −20 °C for at least 28 days [27]. Further the samples were found to be stable in saliva at room temperature for at least 7 days, with a mean relative SD of 6.0%, 8.8% and 8.4% for melatonin, cortisol and testosterone, respectively. The determined SD for long term and short term stabilities were below the interassay variation for melatonin, cortisol and testosterone in saliva. Hence we conclude that storing the saliva samples 7 days at room temperature and three months at −20 °C did not affect the measurement of melatonin, cortisol and testosterone. Hence saliva samples may be used in field studies using self-sampling and returning the saliva samples by mail.

3.4. Circadian rhythms

We tested the developed method in a pilot study of evaluation of circadian rhythms for cortisol, melatonin and testosterone in saliva among four healthy volunteers. They provided six saliva samples during a normal workday and the samples were analyzed by use of the developed method and the results are summarized in Fig. 3. The results showed, in line with clinical studies, the highest concentration of melatonin in morning and the lowest in the evening [53]. Female 1 and female 3 are both self-reported to be morning people and are tired at night. This is reflected in their melatonin samples: Their melatonin level start to rise rapidly already at 20.00. Cortisol showed a morning rise 30 min after awakening for 3 of our 4 volunteers [19] and a fall during the day. This is in accordance with a study done in healthy prepubertal boys also showing these characteristic melatonin and cortisol profiles in saliva [10]. Testosterone showed a tendency to fall during the day for all our volunteers this is in accordance with literature describing the highest testosterone levels in the morning [9]. These results demonstrate that the developed method can be used to monitor the circadian rhythms for all three hormones.

In conclusion, we developed and evaluated a new method for the simultaneous determination of melatonin, cortisol and testosterone within the relevant biological concentration range. The sensitivity of the method ensures that testosterone can be measured in females and that melatonin can be measured during the day. We showed that the method could be used to follow the circadian rhythms of melatonin, cortisol and testosterone in healthy adults. The simplicity, speed and the ability to measure diurnal rhythms in all three hormones, allows us to use this method in future field and clinical studies focusing on psychosocial working environment, including stress and evaluation of circadian disruption.

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