Salivary Melatonin as a Circadian Phase Marker: Validation and Comparison to Plasma Melatonin

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Abstract There are many situations in which it would be useful to know the phase state of the biological clock. It is recognized that measurement of melatonin levels can provide this information, but traditionally blood has been used for the analysis, and there are many problems in extending the measurements into the home or field situations. The aim of this study was to develop and validate a salivary melatonin radioimmunoassay and to compare results obtained against a plasma assay for determining the onset of melatonin secretion. The assay developed was sensitive (4.3 pM) and required only 200 µl of sample. A rhythm in melatonin was detected in saliva, peaking at approximately 120 pM or 30% of the plasma levels. Using an objective criterion for determining the onset of secretion (mean ± 2 standard deviations of three daytime samples), the time of onset was shown to exhibit low intraindividual variability (coefficient of variation = 1.5%-4.3%). The time of onset determined using saliva was significantly correlated with the plasma onset ($r = .70$, $p < .05$). The onsets determined were 22:30 h ± 22 min for the saliva and 21:50 h ± 16 min for plasma for 17 subjects. Similarly, the acrophases of the saliva and plasma melatonin rhythms were significantly correlated. Neither posture alone nor changes in posture affected the calculation of the onset of melatonin secretion using the saliva approach. Very high saliva flow rates induced by citric acid resulted in lower melatonin concentrations compared to the gentle chewing on parafin film. These results firmly establish the use of salivary melatonin measurements for phase typing of the melatonin rhythm in humans.

Key words melatonin, rhythm, saliva, circadian phase

INTRODUCTION

There is now widespread recognition of the central role of the suprachiasmatic nucleus (SCN) in the timing of many important physiological processes. These include the rhythms of sleep-wakefulness, temperature, pituitary-adrenal gland secretions, and the pineal gland hormone melatonin. There is a clear need for a simple, reliable objective measure of the phase of the circadian timing system. A prerequisite would be that the measure not be masked by behaviors such as voluntary sleep (napping) and exercise.

The circadian rhythm of melatonin secretion from the pineal gland has long been considered a useful

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marker because it is essentially directly controlled by the SCN. The only known exogenous modifier of melatonin rhythmicity is environmental light, which entrains SCN rhythmicity and thus all rhythms. Radioimmunoassay (RIA) or mass spectrometric assays of blood melatonin have been used most extensively in carefully controlled clinical and experimental studies. The requirement of multiple blood sampling from indwelling cannulas by necessity confines the blood melatonin testing to the laboratory or special clinics. Urinary melatonin metabolite excretion has been shown to provide a reliable index of pineal gland melatonin production, and a direct RIA is available (Aldhous and Arendt, 1988). The timing of the melatonin rhythm can be assessed using this approach (Deacon and Arendt, 1994a), and collections can be obtained outside normal clinical settings, but a major disadvantage of rhythm assessment using urine is the longer intersample interval of 2 to 4 h that usually is used, which reduces the precision of the assessment of the time of onset of production. Secondary, but nevertheless important, considerations are the need to collect the samples privately, the taboos associated with urine, the need to measure the volume of the void accurately, and possible imprecision in the 6-sulphatoxymelatonin excretion rate resulting from incomplete voiding.

Melatonin has been detected in saliva and has been shown to have a rhythm similar to that of blood melatonin, but because of the low concentrations, assays have been technically challenging. As a body fluid, saliva can be collected noninvasively in good quantity in a short time, and there are likely to be fewer social taboos or restrictions associated with its collection in a wide range of situations.

Despite the acceptance that melatonin can be measured in saliva, there have been no systematic studies of the relationship between the time of onset of the appearance of melatonin in blood and that in saliva. Similarly, there have been no studies on the reliability of salivary melatonin rhythm characteristics with a view to phase typing subjects. In the present study, we measured blood and salivary melatonin levels at frequent intervals during late afternoon through to late morning in a large number of subjects using a new sensitive direct RIA. Objective criteria were used to determine the time of onset of nocturnal melatonin production from both blood and salivary measurements. The test-retest validity of estimates of the time of onset of melatonin production using saliva and blood was assessed together with the impact of posture, change in posture, and salivary flow rate on the estimation of the time of onset of melatonin production.

MATERIALS AND METHODS

For the simultaneous study of blood and salivary melatonin, 17 subjects (8 females and 9 males, mean age 24 years) responded to an advertisement at the University of Adelaide in South Australia. The subjects were screened for general health using a questionnaire and were excluded if they were smokers, had current health or sleep problems, or were taking medication known to affect melatonin production (e.g., β-adrenergic blockers). All subjects gave their informed consent and were compensated for the inconvenience associated with their participation. The studies were approved by the Queen Elizabeth Hospital Ethics of Human Experimentation Committee using guidelines established by the National Health and Medical Research Council of Australia.

All subjects refrained from alcohol and caffeine for 24 h prior to and during the experimental night. On arrival at the Sleep Research Centre at 15:00 h, the subjects brushed their teeth without toothpaste and thoroughly rinsed their mouths with water. Saliva and blood (5 ml) were collected simultaneously from 16:00 until 10:00 h with hourly sampling between 16:00 and 20:00 h and between 02:00 and 10:00 h. Between 20:00 and 02:00 h, samples were collected every 30 min. Blood was obtained from an indwelling cannula in the antecubital vein, whereas saliva was collected during 2 min of chewing a small piece of parafin film. Subjects ate food only after the 18:00- and 07:00-h sample collections, and immediately after eating they brushed their teeth without toothpaste and rinsed their mouths 10 min before the next collection. Water or orange juice was available throughout the study up to 10 min before each sample; in the case of orange juice, the mouth was rinsed with water 10 min before the saliva collection. All subjects remained awake in the supine position throughout the study. The light intensity always was less than 50 lux during the night.

For the reliability studies, 6 subjects (4 females and 2 males, mean age 33 years) collected saliva at home from 19:00 to 23:00 h across 5 consecutive nights. Subjects were instructed to abstain from eating and drinking during the collection period, and they brushed their teeth without toothpaste and rinsed their mouths with water 10 min before the first sample.

For the studies on the effect of posture on salivary melatonin, 6 subjects (5 males and 1 female, mean age 23 years) collected saliva at 30-min intervals at home for at least 3 nights while sitting to determine the time of onset of the salivary melatonin increase. In 5 subsequent trials, the subjects collected saliva at 15-min intervals starting 30 min before the previously determined time of melatonin onset. In the first 3 trials, the samples were obtained while in the sitting, standing, or supine position for the entire collection period. On the 4th night, subjects changed from the standing position to the supine position for 30 min and then stood for 30 min. The 5th night reversed the order of the position changes (i.e., supine to standing to supine).

For the evaluation of the effects of flow rate on salivary melatonin, the 6 subjects used in the posture study collected samples while sitting 30 min before the predicted time of onset, at the predicted time of onset, and 30 and 60 min after the predicted time of onset. Saliva was collected for 1 min by placing plain Salivettes (Sarstedt, Disposable Products, Regency Park, Adelaide) in their mouths without chewing. After 1 min rest and swallowing any residual saliva, another saliva collection was made using citric acid-impregnated Salivettes for 1 min. The Salivettes were preweighed, and flow rate was determined by weighing prior to centrifugation. As a comparison, the saliva flow rate achieved by chewing on parafin film also was calculated from the sitting stage of the posture trial. The saliva was recovered following centrifugation for 10 min at $900 \times g$ in a Beckman J6B centrifuge and was stored frozen until assayed. Preliminary analysis showed that the citric acid in the Salivettes did not interfere with the RIA and that melatonin could be quantitatively recovered from the Salivettes. A further 3 subjects (45-year-old male, 39-year-old male, and 24-year-old female) collected saliva in similar circumstances at 30-min intervals for at least 2½ hours after their melatonin onset.

RIA of Melatonin

Plasma melatonin was assayed using the protocols and reagents from kits obtained from Buhlmann Laboratories AG (Allschwil, Switzerland), with minor modifications. This assay uses the G280 antibody (Earl et al., 1985; Kennaway et al., 1982) first used in a [^{3}H]-melatonin-based RIA and later for a [^{125}I]-2-iodomelatonin-based RIA (Vaughan, 1993). Melatonin was extracted from plasma using small C18 reverse phase columns. Briefly, columns were placed in 10×125 mm glass tubes and sequentially washed by centrifuging for 1 min at $200 \times g$ with 2×1 ml methanol and 2×1 ml water. Standards (reconstituted in phosphate-buffered saline, 0.1 M, pH 7.4, containing 0.5% bovine serum albumin), quality control samples, and plasma samples (0.5 ml) were loaded with a pipette and centrifuged and then sequentially washed with 2×1 ml 10% methanol and 1 ml *N*-hexane. Columns were then placed in clean borosilicate tubes and melatonin eluted by centrifugation with 1 ml methanol. The solvent was evaporated using a heating block held at 37°C and a stream of air. The extract was reconstituted with 1 ml buffer and 400 µl incubated with 100 µl of antibody and [^{125}I]-2-iodomelatonin solutions at 4°C overnight. Bound and free melatonin were separated using a cellulose-immobilized anti-goat second antibody suspension.

Saliva melatonin was assayed by a direct RIA using reagents from the Buhlmann kits. Prior to analysis, previously frozen saliva was thawed and centrifuged for 10 min at $900 \times g$, and the supernatant was decanted and either used immediately or refrozen. Standards (4.3-430 pM) were reconstituted in charcoal-treated (melatonin-free) saliva. Saliva samples, quality controls, and standards (all 200 µl) were incubated with antibody and [^{125}I]-2-iodomelatonin (final volume 600 µl) overnight at 4°C. An anti-goat second antibody was used as already described to separate bound from free melatonin. As a validation step, selected saliva samples were assayed both by the direct assay and following column extraction as already outlined for the plasma samples and a further set of samples assayed at various volumes (200, 150, 100, 50, and 25 µl) to test for parallelism.

Statistics. Data from the plasma-saliva comparison study were analyzed by COSINOR analysis using a program for the Apple II computer provided by Vokac (1984). For this analysis, alpha was set at .001. In all other analyses, alpha was set at .05.

RESULTS

Validation of the Assay

Using a direct RIA, melatonin was detectable in 200-µl samples of saliva with low levels during the day and higher levels at night. The levels ranged from < 16.4 to 102.0 pM (mean ± *SEM*: 33.2 ± 4.8 pM) between 16:00 and 20:00 h. The 1 subject with daytime levels of

Table 1. Repeatability of the determination of the onset of melatonin secretion using saliva across 5 nights with three different methods of calculating the parameter.

Subject	*Method 1*	*Method 2*	*Method 3*
Ath	22:06 (3.4) $n = 5$	20:54 (1.45) $n = 5$	20:42 (2.50) $n = 5$
Vas	20:12 (0.75) $n = 4$	20:24 (0.85) $n = 4$	20:30 (3.70) $n = 5$
Kal	22:42 (0) $n = 2$	21:48 (4.6) $n = 4$	20:42 (4.3) $n = 5$
RK	No onset	22:30 (1.6) $n = 3$	21:12 (1.5) $n = 4$
AT	No onset	21:18 (3.6) $n = 4$	21:12 (2.6) $n = 5$
CV	No onset	No onset	No onset

NOTE: Each data entry shows the mean time of day for the calculated onset with the coefficient of variation of the estimate in parentheses and the number of trials for which the parameter could be determined. Method 1 was based on a threshold of 40 pM, Method 2 used twice the average concentration, and Method 3 used the average plus 2 standard deviations from the mean. Note that for subjects RK and AT, the threshold of 40 pM was not exceeded on any sampling occasion. Subject CV had undetectable melatonin in the saliva at all sampling times.

102 pM was > 3 standard deviations higher than the mean and was identified as an outlier but was included in statistical analyses unless specified. Figure 1 shows the correlation between extracted and direct assays of salivary melatonin for 74 samples from 3 subjects across the day and night ($r = .95$). Because the assay variance is different for the two assays, it was not valid to fit a simple regression line to the data. Nevertheless, it is clear that the data are evenly distributed around the line of equivalence. Nighttime saliva samples from 18 subjects were assayed using sample volumes of 200, 100, 50, and 25 µl saliva (volume made up to 200 µl with charcoal-stripped saliva). The values obtained were 68 ± 13, 67 ± 10, 77 ± 7, and 82 ± 7 pM, respectively, with no significant differences in the concentration of melatonin calculated when the different volumes of saliva were analyzed, suggesting parallelism with the standard curve.

Validation of Procedures for Calculating Melatonin Onset

A total of 6 subjects collected saliva at half-hour intervals between 19:00 and 23:00 h for 5 nights, and melatonin concentration was determined by direct RIA. The onset of melatonin was calculated using three different methods: (1) taking 40 pM as a threshold; (2) taking twice the average of samples collected at 19:00, 19:30, and 20:00 h as the threshold; and (3) taking the mean plus 2 standard deviations above the mean of 19:00, 19:30, and 20:00 h values as the threshold. Table 1 shows the calculated onset times and the associated coefficients of variation. Because 1 subject had salivary concentrations < 4.3 pM on almost every

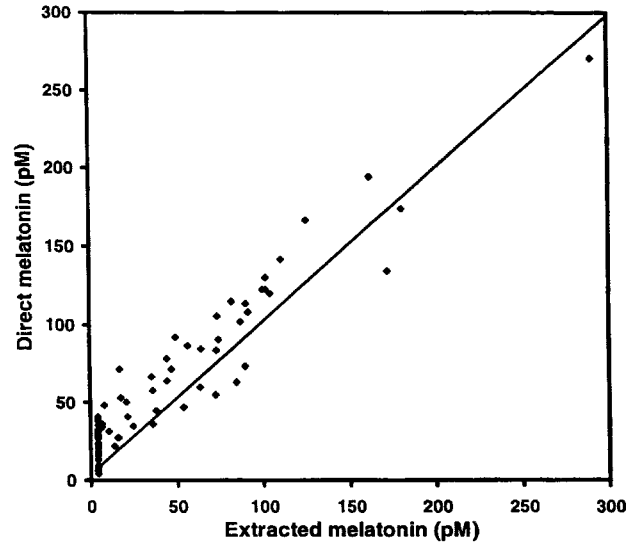

Figure 1. Correlation between direct and extracted saliva melatonin radioimmunoassay. Note that the line is the line of equivalence, not the regression line.

sampling occasion, that subject was excluded. An onset of melatonin secretion could be calculated using Method 1 on all nights for 1 subject, on 4 nights for 1 subject, on 2 nights for 1 subject, and on no nights for 2 subjects. Assessment of onset using Method 2 resulted in 1 missed night for 3 subjects and 2 missed nights for 1 subject. Method 3 allowed the calculation of onset values on all 5 nights in 4 subjects and on 4 nights in 1 subject. The coefficients of variation within subjects were low for all subjects when the onset could be calculated; they averaged 2.4% and 2.9% for Methods 2 and 3, respectively. Of the 5 subjects, 1 (Ath) collected samples over 5 consecutive nights, 5 weeks after the first occasion (Fig. 2). When the mean onset time was calculated with Method 3, it was 20:42 h (coefficient of variation [*CV*] = 1.3%) compared to 20:42 h (*CV* = 2.5%) in the first series.

Comparison of Blood and Saliva Melatonin

Figure 3 shows the melatonin secretory profiles in plasma and saliva for 15 of the 17 subjects (excludes Subject 3 due to elevated baseline and Subject 6 due to failure to detect a nocturnal rise in saliva melatonin). Table 2 shows the individual mean concentrations and their mean plus 2 standard deviations values that were used for determining individual onsets for both plasma and salivary melatonin.

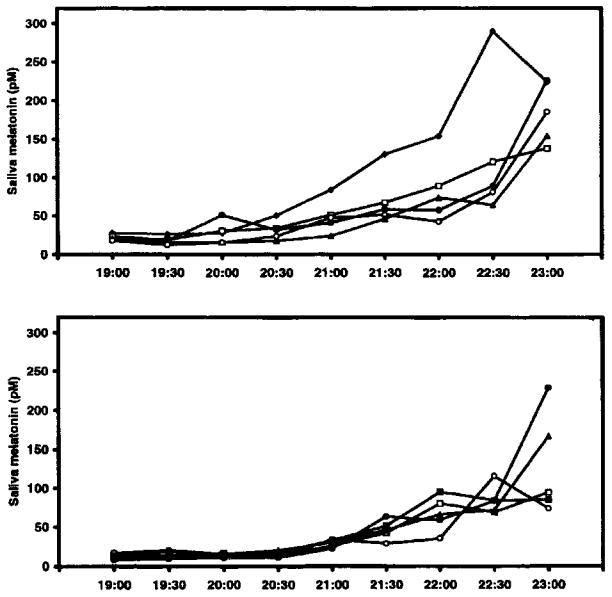

Figure 2. Saliva melatonin profiles for a subject (Ath) determined over 5 successive nights on two occasions 5 weeks apart. The data show the melatonin concentrations in picomolars.

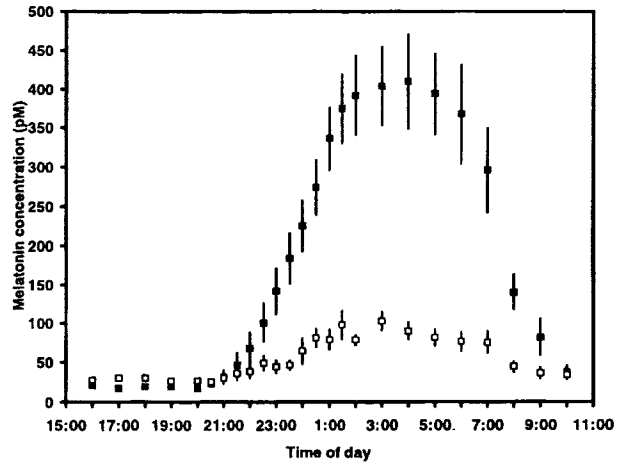

Figure 3. Plasma and saliva melatonin concentrations from 15 subjects. The data are expressed as the mean ± *SEM* picomolars. The plasma and saliva melatonin are represented by the open squares and closed squares, respectively. Note that the data for Subjects 3 and 6 have been omitted (see text).

The thresholds for Methods 2 and 3 ranged from 14.4 to 61.4 pM and from 12.8 to 48.0 pM for plasma and ranged from 32.0 to 98.0 pM and from 21.6 to 78.0 pM for saliva, respectively. When onsets were calculated using the three methods, Method 3 provided onset estimates in all 17 plasma profiles and 16 saliva profiles (including Subject 3), in contrast to the other methods in which onsets could be calculated in 17 plasma profiles but only 13 saliva profiles. Table 3 shows the calculated onset times and the interindividual coefficients of variation, whereas Fig. 4 shows the correlation (r = .70, p < .05) between the onsets calculated for saliva and plasma using Method 3. As a test of the effect of sampling frequency on the determination of the onset, we calculated the melatonin onset in plasma and saliva using Method 3 on the samples collected on the clock hour. The onset determined in plasma was 21:36 h ± 22 min (*CV* = 4.9%), whereas that of saliva was 22:25 h ± 27 min (*CV* = 8.0%), which compared favorably with estimates obtained with more frequent sampling (21:48 and 22:30 h, respectively).

The saliva/plasma ratio was determined in 16 subjects (Subject 3 excluded) from the time of the calculated onset in plasma until 09:00 h. The ratio averaged 0.29 between subjects and did not vary significantly across the night (data not shown).

Evaluation of Other Aspects of the Melatonin Rhythm

COSINOR analysis was possible in 15 of the 17 plasma profiles (sampling was stopped during the night due to cannula failure in 2 subjects well after the nocturnal increase). All plasma profiles could be fitted to sine curves with the percentage rhythm averaging 84.8 ± 2% (range 71%-97%) (i.e., all fits were achieved with a p < .001). For saliva, the percentage rhythm was 58.4 ± 4% (range 10%-81%) (i.e., 1 not significant, 4 others achieving p values between .03 and .004, and the remaining p < .001). In the 14 cases in which the plasma and salivary melatonin acrophases could be compared, there was a significant correlation (r = .55, p = .05). The acrophases for the plasma and salivary melatonin were 03:41 h ± 14 min and 03:40 h ± 20 min, respectively.

When the areas under the rhythm curves were calculated, the values for plasma and salivary melatonin were 1668 ± 197 and 566 ± 95 units, respectively. The amount of melatonin appearing in saliva was thus approximately 30% of that appearing in plasma. Individual onsets, acrophases, areas under the curves, amplitudes, and saliva/plasma ratios are shown in Table 4. Multiple correlation analysis showed that the plasma and salivary melatonin onsets (calculated using Method 3) were significantly correlated, as were

Table 2. Mean plasma and salivary melatonin concentrations for the subjects in the study and their dim light melatonin onset thresholds calculated using Method 3 (picomolars).

	Plasma		*Saliva*	
Subject Number	*Mean Plasma Melatonin*	*Method 3 Threshold*	*Mean Salivary Melatonin*	*Method 3 Threshold*
1	16	24	24	28
2	28	30	48	78
3	20	26	102	138
5	30	36	24	44
6	14	20	32	38
7	12	16	28	36
8	10	10	16	22
9	12	12	34	44
10	8	14	32	40
11	26	46	28	34
12	10	12	20	22
13	20	22	20	30
14	20	34	28	34
15	12	22	28	28
16	18	22	20	26
17	28	48	50	72
18	28	32	32	40
Mean	18.4 ± 1.8	25.0 ± 2.8	33.2 ± 4.8	44.0 ± 6.8
			29.0 ± 2.4	39.0 ± 4.0

NOTE: The mean concentrations were calculated using samples collected at 19:00, 19:30, and 20:00 h. The Method 3 thresholds represent the means ± 2 standard deviations. Note that for the saliva, Subject 3 had a baseline more than 3 standard deviations from the mean. The lower values in the group mean row exclude this subject.

Table 3. Mean calculated onset of the appearance of melatonin in plasma and saliva for all subjects using three different methods of calculating the parameter.

	Plasma	*Saliva*
Method 1	22:18 (5.2) $n = 17$	23:30 (6.4) $n = 13$
Method 2	22:12 (5.2) $n = 17$	01:03 (6.3) $n = 13$
Method 3	21:48 (5.2) $n = 17$	22:30 (6.6) $n = 16$

NOTE: The data are the average times of the day for the melatonin onset calculated using the three methods. Also shown for each data entry are the coefficient of variation for the group in parentheses and the number of subjects for which the parameter could be calculated.

plasma onset and plasma acrophase as well as saliva onset and plasma acrophase. Simlarly, the plasma acrophase was significantly correlated with the saliva onset ($r = .64$) and plasma onset ($r = .83$).

Effect of Posture on the Determination of Melatonin Onset

The 6 subjects who participated in the posture study had a mean salivary melatonin onset at 22:21 h ± 45 min in the initial trial. When the onsets were calculated following the sessions of sitting, standing, lying, and changing posture around the time of the predicted onset, there was no significant effect of posture on the calculation of the parameter (Fig. 5).

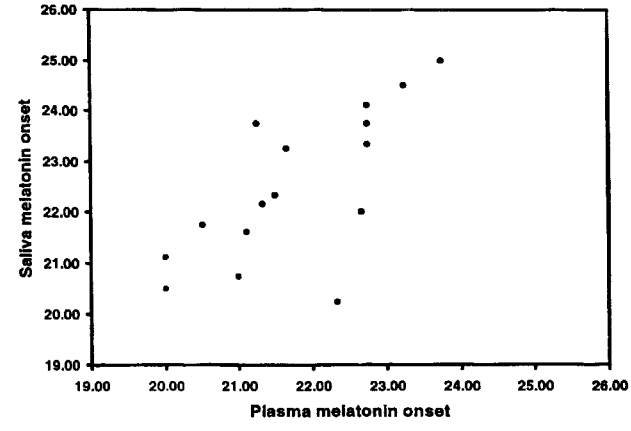

Figure 4. Relationship between the plasma melatonin onset and the saliva melatonin onset as determined by Method 3.

Effect of Flow Rate on Salivary Melatonin Levels

When the same 6 subjects subsequently conducted a trial with plain and citric acid-impregnated Salivettes for saliva collection, there were significant differences in both the salivary flow rate and the saliva melatonin concentrations. The citric acid Salivettes resulted in a 65% to 90% increase in saliva flow compared to the plain Salivettes (3.0 ± 0.3 ml/min vs. 1.7 ± 0.3 ml/min). Whereas a significant increase in salivary melatonin occurred with time using the plain Salivettes, no such increase in salivary melatonin was observed within 60 min of the expected time of the rise following citric acid stimulation. These results should be compared to the increase in melatonin concentration observed in the sitting stage of the posture study in which saliva was collected by chewing on parafin film. The flow rate in that trial was not significantly different (1.2 ± 0.1 ml/min) from the plain Salivette-induced flow (1.7 ± 0.3 ml/min), and melatonin levels increased significantly across the 2 h of the test. To test whether salivary melatonin could be detected at all using the citric acid-treated Salivettes, 3 subjects collected saliva for an extended period in the same way as just described Melatonin could be detected in highly stimulated saliva (data not shown), but onsets calculated were later than

Table 4. Plasma and saliva circadian rhythm characteristics for individual subjects.

Subject Number	*Saliva Onset*	*Plasma Onset*	*Saliva Acrophase*	*Plasma Acrophase*	*Saliva AUC*	*Plasma AUC*	*Saliva Amplitude*	*Plasma Amplitude*	*Saliva/Plasma Ratio*
1	21:36	21:07	03:27	—	358	—	37.2	—	0.24
2	21:07	20:00	01:06	02:08	766	1840	96.4	344.8	0.40
3	23:20	22:44	06:23	—	1904	—	141.2	—	—
5	00:30	23:15	05:35	04:54	576	3232	64.8	673.6	0.16
6	—	22:45	—	04:55	250	998	—	167.4	0.24
7	23:45	21:15	02:18	03:02	174	1533	30.8	345.8	0.16
8	23:15	21:40	03:53	04:28	198	1025	17.6	174.4	0.20
9	20:15	22:20	04:44	04:14	814	837	52	156	0.72
10	24:07	22:45	03:55	04:12	388	1338	30	279.8	0.29
11	22:00	22:40	03:18	03:23	476	1892	63.2	411.8	0.20
12	21:45	21:00	02:13	02:57	324	1318	37.6	256.8	0.21
13	22:20	21:30	03:41	03:31	598	3306	106.4	724.8	0.17
14	23:45	22:45	03:19	03:25	384	814	48.2	163.8	0.43
15	20:30	20:00	03:44	02:40	538	1416	51.6	243.2	0.34
16	21:45	20:30	05:24	03:12	404	2094	20.6	386.8	0.18
17	01:00	23:45	03:10	05:09	680	1384	70.2	208.4	0.34
18	21:10	21:20	02:48	03:49	692	1999	112.4	366	0.36
Mean	22:30 h ± 22 min	21:50 h ± 16 min	03:40 h ± 2 min	03:41 h ± 14 min	566 ± 95	1668 ± 197	62 ± 9	326 ± 44	0.29 ± 0.05

NOTE: AUC = area under the curve. The data show the individual times of onset, times of acrophases, AUCs (arbitrary units), and rhythm amplitudes (picomolars) calculated from saliva and plasma melatonin measurements. The saliva/plasma ratio for each subject is based on measurements between the onset time and 09:00 h. The mean value ± *SEM* also is shown for each parameter.

those determined using other collection procedures because of the low concentrations.

DISCUSSION

A major aim of our study was to validate the use of salivary melatonin measurements for determining the time when active melatonin secretion commenced at night in subjects kept in dim light. Lewy and colleagues (1985) have pioneered the use of what they called the dim light melatonin onset (DLMO) for determining the phase of the melatonin rhythm. They used the gas chromatography mass spectrometry melatonin assay for plasma to determine the time that melatonin concentration exceeded 10 pg/ml (43 pM) and used this as their phase marker. Although it has been used in several articles (Lewy et al., 1987; Lewy and Sack, 1989), we have been unable to find any information regarding the test-retest variability of the DLMO approach. In our assessment of the salivary melatonin assay for phase typing, we first examined three commonly used approaches for detecting increased hormone levels in the presence of an unstable baseline. In addition, we conducted test-retest trials. In our Method 1, we used a rigid threshold value (40 pM) above which the melatonin concentration had to remain elevated to give the phase position. This was similar to the approach used for the original plasma DLMO (Lewy et al., 1985). This appeared reasonable for plasma, allowing onsets to be determined for all subjects, but it failed to detect an increase in melatonin in the saliva of 4 subjects due to the levels being continuously above or below the threshold value. Similar results were observed for Method 2, which used a threshold of twice the average of baseline values. Method 3 was more successful, allowing onsets to be determined for 16 of 17 subjects from their salivary melatonin results. We believe this method also is statistically superior because it relies on a melatonin measurement being significantly different from the baseline levels as well as compensating for individual differences in the baseline. The intraindividual coefficients of variation of the derived onset times were very low for the saliva approach (1.5%-4.3%). When the intraindividual coefficients of variation of the determined onset times were calculated for the subjects collecting on 5 successive nights, they ranged from 1.5% to 4.3%. Thus, it is clear that onset estimates determined using salivary melatonin are highly repeatable.

There was a significant correlation between onsets determined from plasma and saliva measurements using Method 3. The actual time of the salivary melatonin onset was significantly later than that determined in plasma (40 min), but this probably is of little practical importance. The acrophases calculated from the COSINOR analysis also were significantly correlated, and the group means for the two acrophases were almost identical. Although interesting, it might

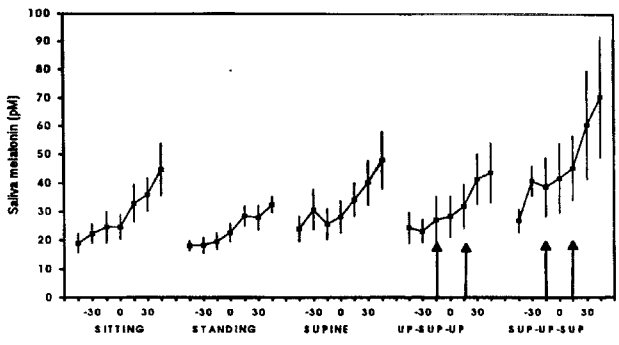

Figure 5. Effect of posture and change in posture on the saliva melatonin levels in 6 subjects. Subjects remained sitting, standing, or supine or changed from sitting to standing to supine to standing and from sitting to supine to standing to supine at times indicated by the arrows. The data are the mean ± *SEM* picomolars.

be said that the determination of a salivary melatonin acrophase is of little practical use as a phase marker because subjects must remain awake to collect the samples, unlike the case of blood collections.

When the areas under the secretion curves were compared between plasma and saliva, it was clear that the amount of melatonin appearing in saliva was approximately 30% of that appearing in plasma. Interestingly, there was no correlation between these two measures. Similarly, the rhythm amplitudes calculated in the COSINOR analysis for saliva were approximately 25% of those for plasma and were not correlated. This observation of marked interindividual differences in the saliva/plasma ratio (range 0.16-0.72) raises some interesting physiological questions as well as strengthens the validity of using the statistical approach of Method 3 against the preset threshold approach. It has been presumed that the lower melatonin levels in saliva reflect plasma binding, but there has been remarkably little research addressing this question. A quarter of a century ago, Cardinali and coworkers (1972) studied the binding of [3H]-melatonin to rat and human plasma proteins using equilibrium dialysis procedures and determined that melatonin was bound with low affinity to albumin-like proteins. The binding was temperature dependent (78% bound at 4°C and 61% bound at 37°C) and was not modified by related methoxyindoles. Later, a similar value for the binding of [3H]-melatonin to blood proteins was reported (Pardridge and Mietus, 1980). In previous studies that addressed the saliva/plasma ratio, it was found to vary from 0.21 to 0.91 (Laakso et al., 1990; McIntyre et al., 1987; Miles et al., 1985a, 1987, 1989; Nowak et al., 1987; Vakkuri, 1985; Vakkuri et al., 1985). Why some subjects retain up to 90% of the melatonin in the plasma is not at all clear. Similarly, it is not known what the physiological consequences of high and low plasma binding might be, although in the case of many other hormones and drugs, it is the free fraction that is able to act at target sites. Whether the binding of melatonin with low affinity to plasma proteins is of physiological consequence in this respect is not known, but it clearly is an area deserving study.

Deacon and Arendt (1994b) reported a potential masking phenomenon for melatonin levels in blood and saliva. They showed that the rapid redistribution of fluid when moving from the supine to the standing position decreased the concentration of melatonin in plasma and saliva, a masking effect that was reversed when the subjects resumed the supine position. Under most experimental circumstances, subjects can be confined to one position; however, in ambulatory situations such as shift work studies, changes in position might be expected to contribute to a noisy melatonin profile. We considered it important, therefore, to evaluate the impact of changes in posture around the time of onset of melatonin secretion on the calculation of the time of onset using saliva. During the time that melatonin levels were increasing in the saliva, we observed no consistent effect of posture change on the calculated onset time.

In earlier studies on salivary melatonin, authors often stated that they collected saliva without stimulation (Koller et al., 1994; Laakso et al., 1990, 1994; Miles et al., 1985a, 1985b, 1987; Nowak et al., 1987), whereas others collected saliva induced by chewing on parafin film (Deacon and Arendt, 1994a, 1994b; Laakso et al., 1993) or rubber bands (Miles et al., 1989) and yet others gave no details (Deacon et al., 1994; English et al., 1993; Harma et al., 1994; McMillen et al., 1993; Stokkan and Reiter, 1994; Vakkuri, 1985). A review of the literature uncovered only one formal study on the effect of flow rate on melatonin concentrations in saliva (Miles et al., 1989). These authors reported a comparison of melatonin concentration from "unstimulated" saliva collections and saliva stimulated by chewing on rubber bands. Unfortunately, the method of collection of the unstimulated saliva was not stated, and no actual flow rates were reported in the article. The authors concluded, however, that flow rate did not affect the melatonin levels. We attempted to ad-

dress this question by comparing results obtained by chewing on parafin film (stimulated saliva) with those obtained using plain Salivettes without chewing (unstimulated saliva) and citric acid-treated Salivettes without chewing (stimulated saliva). Remarkably, the flow rates obtained with both the chewing and plain Salivettes were not significantly different, whereas the citric acid treatment increased saliva flow by almost twofold. It would appear that placing the Salivettes in the mouth was in itself a stimulus for saliva flow. The melatonin profiles for the two former procedures were similar, whereas when citric acid Salivettes were used, the appearance of melatonin in the saliva apparently was delayed as a result of lowered melatonin concentrations. A further trial extending the length of collections gave similar results. These results suggest that the transfer of melatonin across the salivary cell membranes is lowered following citric acid stimulation. It is difficult to explain how this might occur other than to suggest that the rate of water transferred from the plasma exceeded the rate at which the melatonin could dissociate from the albumin-like binding protein. We believe the phenomenon is of little practical importance, however, because the flow rates achieved with citric acid clearly were much higher than those achieved by even reasonably vigorous chewing of parafin film. We do not recommend, however, that the citric acid-impregnated Salivettes be used for melatonin analysis.

CONCLUSIONS

We have developed and validated a direct RIA for melatonin in saliva that has proved to be useful for determining the onset of melatonin secretion with a high level of precision. The determination of the onset of secretion was not affected by posture or changes in posture. Salivary melatonin levels were affected by very high salivary flow rates induced by citric acid. This assay and the methods of determining the onset of secretion should prove useful in a wide range of studies in which monitoring of circadian rhythmicity is required.

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