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Circadian Rhythms of Histatin 1, Histatin 3, Histatin, 5, Statherin and Uric Acid in Whole Human Saliva Secretion

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Abstract

The circadian rhythms of histatins 1, 3, 5, of statherin and uric acid were investigated in whole human saliva. Histatins showed a rhythm approximately synchronous with salivary flow rate (acrophase around 5 pm), the higher amplitude pertaining to histatin 1 (about 50% of the mesor). Uric acid showed a large rhythm asynchronous with flow rate and histatin concentrations (4.4 ± 1.4 am). Statherin did not show a significant circadian rhythm on five of six volunteers. This finding confirms that the secretion route of statherin is different from that of histatins.

Keywords: Human saliva, peptides, histatin, statherin, circadian rhythms, urate.

Introduction

Oral fluid represents an easily available medium suitable for clinical studies and for the diagnosis of a widening range of diseases (Mandel, 1993). Screening for the systemic presence of infectious disease (Mortimer & Parry, 1988; Granstrom et al., 1988), differential diagnosis of salivary gland diseases (Tishler et al., 1997), caries (Dowd, 1999) and periodontal diseases (Rudney et al., 1993), as well as monitoring of illicit drug abuse (Schramm et al., 1992) are today common clinical practices performed on salivary specimens. Recently, we have developed a high-performance liquid chromatography (HPLC) method which allows the contemporaneous determi-

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nation of salivary histatin 1, histatin 3, histatin 5, statherin as well as uric acid (Castagnola et al., 2001). The concentration of selected salivary components might give interesting information on anomalous oral and/or systemic conditions (Bercier et al., 1999), on the secretion gland (Dawes & Ong, 1973), age and diet (Salvolini et al., 1999) and during pregnancy (Laine et al., 1988). Since it is well known that composition of human saliva is also dependent on physiological rhythms (Oberg et al., 1982) a knowledge of the range of concentration as a function of time of collection is advisable. For this reason, we have considered useful to investigate circadian rhythms of histatin 1, histatin 3, histatin 5, and statherin in order to establish if they are synchronous with respect to the rhythms of other salivary components and flow rate.

Materials and Methods

Reagents and instruments

All reagents were analytical grade purchased either from Merck (Darmstatd, Germany), Carlo Erba (Milan, Italy) or Sigma-Aldrich (St. Louis, MO, USA). Chromatographic eluents were from Carlo Erba (Milan, Italy). Standard of histatin 5 was purchased from Sigma-Aldrich. Standards of uric acid and tyrosine were obtained from Merck. The HPLC apparatus was a Beckman (Palo Alto, CA, USA) Gold 125S solvent module equipped with a diode array 168 detector and Gold Noveau software. The chromatographic column was a Hewlett-Packard (Palo Alto, CA, USA) Hypersil BDS-C_{18} with 3 µm particle diameter (column dimensions 100 × 4 mm) protected by a guard column of ODS Hypersil resin (5 µm; 20 × 2.1 mm). Matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was performed by a PE-Biosystems Voyager System 1167 (PerSeptive Biosystems, Foster City, CA, USA).

Specimen collection

The six informed (male) donors were advised regarding a common standardized specimen collection procedure. Collection times were established according to a schedule that programmed sampling with, at least, two hours intervals. All the specimen collections were performed during spring, under similar light exposition. Donor avoided drug delivery at least a week before specimen collection. Coffee and tea were omitted from the diet at least 24 hours before specimen collection. Concerning other physiological variables, donor was invited to maintain a normal life-style, with three meals for day, without smoking. Nocturnal samplings were performed at home, awaking the donor for few minutes for the specimen collection and for the measurements of flow-rate. Saliva specimens were collected at the base of the tongue with a small plastic aspirator. The saliva was immediately collected in a plastic tube and 80 mmol/l phosphate buffer pH 2.2 was added to the sample (1 : 1, v/v). Samples were stored at –20°C until analyzed. Before the analysis samples were thawed at room temperature and centrifuged at 8000 g, the precipitate was discharged and the upper solution was analyzed by HPLC method.

Salivary flow rate was determined from the weight of saliva secreted in 1 minute during any sample collection, by using a pre-weighted cotton roll (salivette; Saersted) placed under the tongue.

HPLC and MALDI-TOF-MS analysis

The following solutions were utilized: (eluent A) 80 mmol/l phosphoric acid, pH 2.50; (eluent B) acetonitrile/eluent A (80 : 20, v/v). Gradient development was as follows: 0% B for 1 min, linear gradient from 0 to 75% B in 45 min, linear gradient from 75% to 100% B in 1 min, 100% B for 8 min, linear gradient from 100% to 0% B in 1 min. The flow-rate during the whole gradient development was 1.0 ml/min. The DAD window was established between 190 and 350 nm. The volume injected corresponded to 50 µl for analytical purposes and 100 µL for semi-preparative purposes. The peaks attributed to histatins 1, 3, 5 and statherin, according to spectral properties and polarity criteria, were collected, lyophilized and submitted to MALDI-TOF-MS analysis. The mass determination confirmed peptide attribution within the limits of MALDI-TOF-MS experimental error.

The quantification of histatins 1, 3, 5 and statherin was carried out using the peak area at 276 nm in comparison to standards of tyrosine and on the knowledge of the tyrosine content of salivary peptides, as previously described (Castagnola et al., 2001).

Statistical treatment of data

Experimental data were analyzed according to the usual parametric function (cosine wave, Dawes, 1972):

$$C_t = C_m + A \cdot \cos\left(\frac{2\pi t}{\tau} + \varphi\right)$$

In the function, C_t and represents the concentration of the analyte under investigation at time t, C_m (mesor) the mean concentration of the analyte over the period investigated, A the amplitude of rhythm, τ the period under study (24 h, 360°) and φ the phase angle of the acrophase (time of maximal secretion). Mesor, amplitude and φ values were determined by parametric best fits using minimization procedures.

Results

Previous studies on salivary rhythms have shown that the concentration of some salivary components is affected by sampling when frequent saliva collections are performed (Ferguson & Botchway, 1980). For this reason in the present study subsequent samplings were performed with an interval of at least two hours.

A typical chromatographic separation utilized for the quantification of salivary histatins 1, 3, 5, statherin and uric acid is reported on Figure 1. Analysis was performed at 276 nm in order to reduce the interference deriving from other salivary components

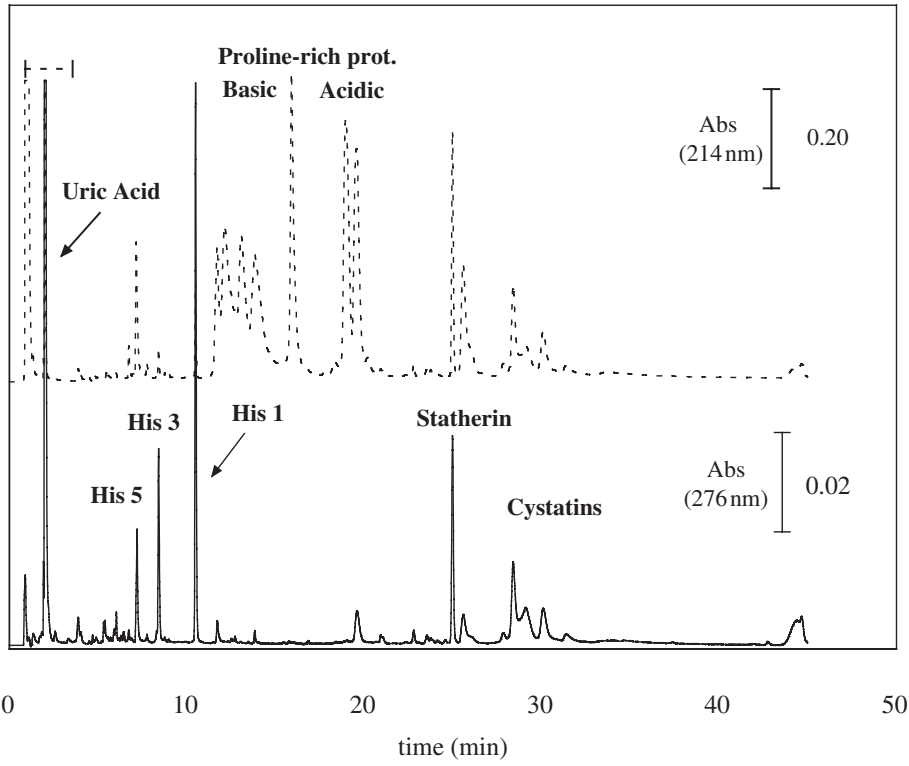

Figure 1. Typical high-performance liquid chromatography pattern, utilized for the determination of the concentration of histatin 1, 3, 5, statherin and uric acid of a human healthy subject. The use of diode-array detection allowed, for the quantification of these salivary components, the selection of a detection wavelength at 276 nm. In the pattern the peaks pertaining to cystatins were also detectable (Castagnola et al., 2001). The dashed line shows the HPLC pattern obtained at 214 nm, where the peaks pertaining to proline-rich proteins were also evidenced. For other information on the separation see the methods section.

and to achieve a satisfactory quantification of salivary peptides on the basis of standards of tyrosine (Castagnola et al., 2001).

In Figure 2 the circadian rhythm of the concentration of histatin 1 (close circles) measured in different saliva samples of one subject collected during 24 hours is reported. Experimental data were fitted with the cosinor function (continuous line) in order to determine mesor, acrophase and amplitude, which characterized the circadian rhythm of this peptide. Salivary flow rates showed circadian rhythms in a good agreement with those reported in literature (Dawes, 1972), being the maximal and the minimum salivary flow rate approximately at 18 pm and at 6 am, respectively (Fig. 3 and Table 1).

The parameters describing the circadian rhythms of histatins 1, 3 and 5, statherin and uric acid on six male healthy volunteers are reported in Table 1 and Figure 3.

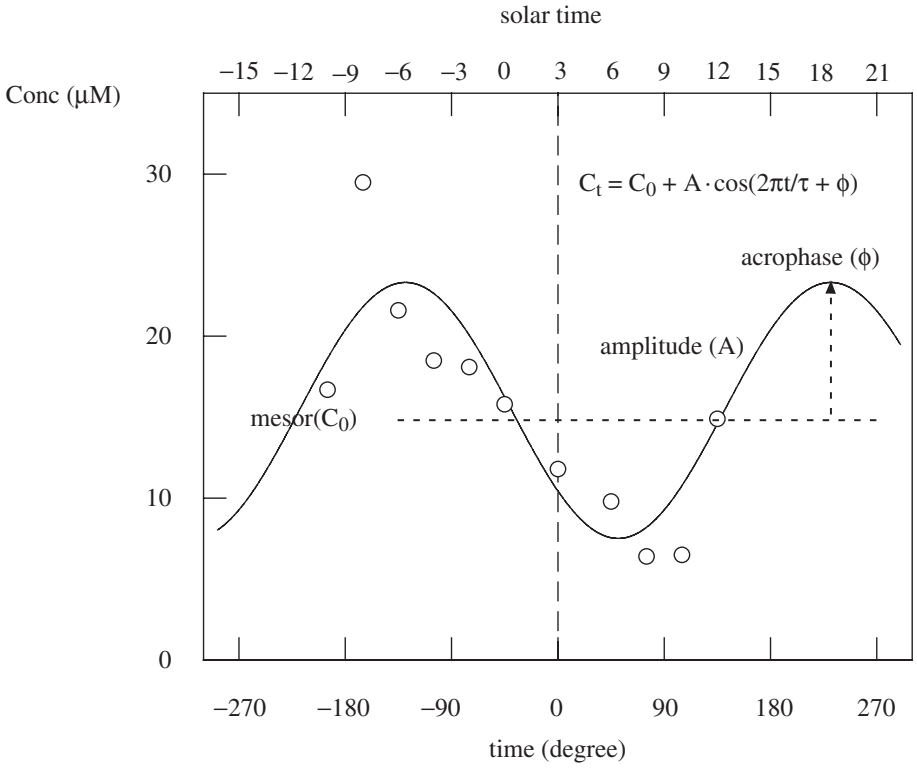

Figure 2. Circadian rhythm measured for the concentration of histatin 1, on one of the healthy volunteers submitted to the study. The parameters of the cosinor analysis (Dawes, 1972) are indicated.

Whereas histatins showed a secretion synchronous with the flow rate, statherin did not show an appreciable rhythm of secretion and uric acid showed a rhythm asynchronous with respect to salivary flow rate. In fact, histatin acrophase is positioned approximately at 17 pm, whereas that of uric acid is roughly positioned at 5 am. The amplitude of the histatin and uric acid rhythms are large (Table 1) reaching a value of about 50% the mean value of the interval (mesor) for histatin 1. Thus, oscillations in the concentration of histatin 1, originated by the circadian rhythms, can provide differences in the results that are more than two times greater than the standard deviation observed on the mesor value. The amplitude of the rhythm is greater than the standard deviation on mesor also for histatin 5 and uric acid concentration, whereas is of the same order of magnitude for histatin 3 and flow rate. As far as the concentration of statherin is concerned, only one of six volunteers showed a slight rhythm, synchronous with the flow rate, but these slight oscillations were not observed in others. Moreover, the standard deviation of mean statherin concentration is small with respect to that measured on the other salivary components under study.

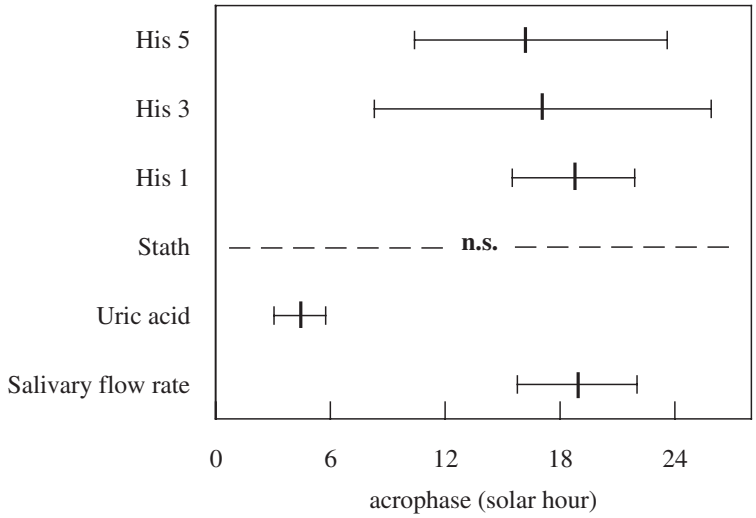

Figure 3. Acrophase measured for the salivary concentration of histatin 1, histatin 3, histatin 5, statherin and uric acid. The measured acrophase of salivary flow rate is also reported. The acrophase interval was established according plus/minus one S.D.

Table 1. Parameters of the cosinor analysis of circadian rhythms on histatins (1, 3, 5), statherin, uric acid and salivary flow rate (n = 6).

Salivary component	mesor	amplitude	acrophase (hour)
Histatin 5 (μM)	15.4 ± 3.8	5.5 ± 2.4 (35.8%)	17.0 ± 6.6
Histatin 3 (μM)	9.8 ± 3.7	2.8 ± 1.1 (28.2%)	17.1 ± 8.8
Histatin 1 (μM)	11.7 ± 2.8	5.8 ± 1.9 (49.3%)	18.7 ± 3.2
Statherin (μM)	8.0 ± 1.9	n.s.	n.s.
Uric acid (μM)	306 ± 63	109 ± 40 (35.4%)	4.4 ± 1.4
Flow rate (mL/min)	0.36 ± 0.12	0.17 ± 0.09 (47.2%)	18.9 ± 3.1

The intervals are reported plus/minus one S.D.

Discussion

Histatins and statherin are small peptides present in human saliva (Oppenheim et al., 1988; Raj et al., 1992). These peptides have a peculiar structure, histatins having high histidine content and statherin high tyrosine content. In human normal saliva statherin is usually present as a unique component, whereas histatins comprise at least twelve peptides of different dimensions. However, ten are probably originated by the action of proteases from the two principal ones, histatin 1 and histatin 3, (Payne et al., 1991; Xu et al., 1993) and only histatin 5 is usually present at a concentration comparable

with that one of the two formers (Sugiyama & Ogata, 1993). The interest for these peptides is growing. In fact, histatins have probably a relevant role in the non-immune defense of oral cavity, particularly devoted against fungal attack (Tsai & Bobek, 1998), while statherin has a relevant role in the homeostasis of the oral calcium (Hay et al., 1984). The analysis of circadian rhythms of these peptides can be useful in order to understand how these peptides explicate their function in the oral cavity. Moreover, since a connection between their concentration and oral and/or systemic diseases was until now not clearly established (Rudney, 1995; Tsai & Bobek, 1998), the rhythm study is essential to obtain information on the normal range of concentration. The results of the present study indicate that histatins concentration show a large rhythm, being the amplitude of histatin 1 and histatin 5 largely greater than the standard deviation computed on the mesor. The same result was also obtained for uric acid concentration, although its rhythm was asynchronous with flow rate. On the other hand, whereas the amplitude of the rhythm of histatin 3 was on the same order of magnitude of the standard deviation of the mesor, statherin seemed to have not a significant rhythm. A common ancestral gene seems to be responsible of the sequence similarities of these peptides (Dickinson et al., 1987). However, whereas the biosynthetic pattern of histatins has been studied, including packaging in acinar cells and a spread distribution throughout the granule (Takano et al., 1993), that one of statherin is unknown. Our data strongly suggested that the route of statherin secretion was different from that one of histatins. In this respect, whereas histatin concentrations showed to correlate among them, no significant correlation between statherin and histatins concentrations was observed (Castagnola et al., 2001). Jensen and colleagues (Jensen et al., 1994) suggested that histatins and statherin are synthesized and packaged differently within the acinar cells. It should be clearly taken into account that the submandibular and sublingual glands are the major responsible for resting salivary secretion, whereas parotid glands are the major responsible for stimulated secretion, hence the gland contribution to salivary flow rate changes during the sampling period (Dawes & Ong, 1973). Therefore, different balancing between gland secretions during the day can account for the absence of a sensible statherin rhythm with respect to histatin rhythm. In this respect, further studies are required in order to elucidate statherin packaging and secretion route.

Temporal rhythms of some components of human oral fluid have been studied, whereas those of histatins and statherin have not. The classical studies of Dawes (Dawes, 1972; Dawes, 1975) and Ferguson's group (Ferguson & Botchway, 1979) established the principal temporal characteristics of salivary secretion. Apart slight variations on acrophase deriving from many factors, not least the social behaviour of the respective country, the secretion of salivary components could be divided in three principal groups. The first group comprises the salivary components synchronous with oral temperature and flow rate, which show an acrophase approximately in the afternoon, the second one comprises the asynchronous components, whose acrophase is in the early morning. The third group includes the salivary components that do not show a sensible rhythm. The secretion of proteins usually pertains to the first group, as observed for histatins. On the other hand enzymatic activities, such as observed in this study for statherin, usually seem to be linked to the third group. Such an example,

salivary lysozime (Richter et al., 1980) and kallikrein (Jenzano et al., 1987) did not reveale sensible circadian rhythm.

Uric acid pertains to the second group. Owen-Smith and colleagues (Owen-Smith et al., 1998) already observed the increase of nocturnal salivary urate. They observed that salivary urate concentration increased significantly in gout and it was significantly related to blood urate. However, whereas blood urate did not show diurnal variation, the salivary urate increased during the sleep. This should depend on a dilution of urate concentration at high salivary flow rate. In fact, an inverse dependence between urate concentration and salivary flow rate has been observed in this study (Fig. 4). Salivary urate is part of a pool of antioxidant substances, including salivary thiocyanate and glutathione (Zappacosta et al., 1999). The interplay between these salivary components and salivary peroxidases action should be responsible for the defense of oral cavity towards oxidative stress. It is interesting to note that the maximal uric acid concentration, more than two times greater that the minimal values, was measured during the night, i.e., at the minimum of the salivary flow rate. A maximal antioxidant salivary potential could be necessary during the night, when the acidity of the oral cavity, sodium, chloride and carbohydrate secretions are at their maximum (Ferguson & Fort, 1974; Ferguson & Botchway, 1980).

Concerning the time of specimen collection, this study indicates that, such as for many others constituent of oral fluid, the normal range of concentration for histatins and uric acid must referred to a precise, accorded schedule of temporal collection.

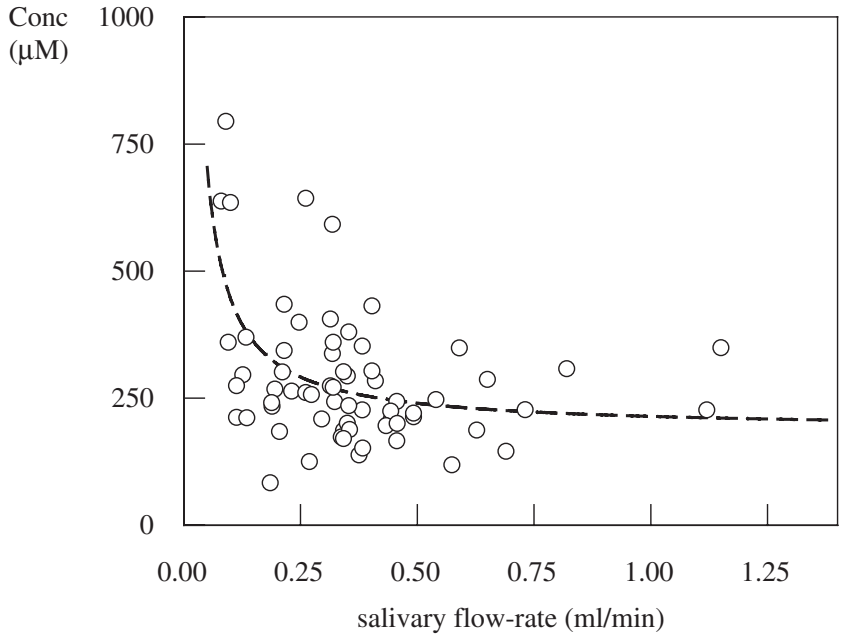

Figure 4. Relationship observed between salivary urate concentration and flow rate.

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