

# Salivary glucose concentration and daily variation in the oral fluid of healthy patients

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## Summary

**Aim.** The aim of this study was to determine the concentration and the variations of salivary glucose in healthy patients who were sampled at five different intervals during the day.

**Materials and methods.** Samples of un-stimulated saliva have been collected from 21 healthy patients using the drooling technique and they were divided into two categories. In the first category, patients were asked not to toothbrush their teeth in the 8 hours prior to collection of the salivary sample. Patients in the second category were instead asked to toothbrush 90 minutes before the collection of the first sample of saliva. The glucose concentration was measured in all patients via an enzymatic spectrophotometry. Patients have been selected following a strict inclusion criteria, which included <5% of plaque presence and according to the plaque index and a total absence of oral disease that could possibly interfere with sample taking or oral fluid analysis. The average age of patients was 22,4±2,6 years old of which 45% were female and 55% were male. Samples were collected five times between 8 am and 12 pm, before and after breakfast. Data was statistically analyzed using the Skewness/Kurtosis Test, Shapiro-Wilk Test, Kruskal Wallis Test and Linear Regression Model, considering values of  $p < 0,05$  to be significant.

**Results.** The average rate of un-stimulated salivary flow was  $0,53 \pm 0,21$  ml/min. There were no significant differences between salivary glucose values and salivary flow in female and male patients. However, we observed a typical trend which recurred for each patient and resembled a "pseudo-glycemic curve".

**Conclusions.** The collected data suggests that glucose concentration results did not show statistically significant differences ( $p > 0,078$ ) which could however be due to the little number of patients assessed, nonetheless there is a trend, recalling a "pseudo-glycemic curve" not referable to changes in flow rates, probably due to glucose catabolism and shunt.

**Key words:** salivary glucose, saliva, oral hygiene, oral fluid, spectrophotometry.

## Introduction

Oral fluid is an organic fluid, easy to collect and to preserve, which may give us plenty of information about the presence of systemic and local diseases via non-invasive sampling methods. It is constituted by a complex chemical milieu of teeth and oral soft tissues, consisting mainly on water, essential electrolytes, glycoproteins, antimicrobial enzymes and numerous other important constituents like glucose (1, 2). In the last decade the concentration of many biomarkers present in saliva has acquired increasing importance possibly showing the presence of systemic illness and reflecting the metabolic, nutritional, immunological, hormonal, emotional and neurological state of the patient (3-5).

Many articles have been written regarding the role and presence of glucose in oral fluids such as saliva and gingival crevicular fluid (GCF) in diabetic patients. However, little efforts have been made to assess healthy patients. Above all, there are no articles that describe repeated sample taking at different intervals of the day (10-14).

Glucose is a small molecule (180 DA) that diffuses through blood vessel membranes and passes through blood serum into GCF and salivary ducts to finally reach the oral cavity (15). Several studies assert that salivary glucose levels are not related to glycaemia, even if the oral fluid of diabetic patients presents higher levels of glucose than those in healthy controls (14-17). Sampling oral fluid is easy, non-invasive for patients and safe for clinicians, However, several authors do not agree on the results obtained from such analyses,

due to the different available protocols in sampling procedures and the different analytical techniques. Ramsaier et al. and Kinney et al. report using one of the most repeatable and simple procedures to sample oral fluid: the drooling technique (2, 18). Many authors sampled oral fluid in a specific range of time and patients were instructed not to brush their teeth, eat, drink or smoke 2-3 hours before the time of saliva sample collection, but none of them have assessed a plaque index score and/or oral health evaluation. This is essential as plaque residuals may play an important role in salivary glucose concentration (19-21). Based on these premises, the goal of the present study is to evaluate the presence and therefore compare salivary glucose concentration in healthy young individuals with excellent oral care. We also intend to analyze unstimulated oral fluid samples taken at different times before and after a standardized breakfast, comparing two different protocols; with tooth brushing (90 min before first sample taking) and without tooth brushing (8 hours from the last domiciliary oral hygiene care).

## Materials and methods

Thirty-seven patients who maintained all permanent teeth and had no salivary dysfunctions were included in the study. Patients were trained for 6 months on how to carry out effective oral domiciliary hygiene care. They were monitored until they reached a Plaque Index score, which was less than 5% according to the Silness and Loe index. Of the thirty-seven patients assessed, only twenty-one of them have managed to reach a 5% plaque level within the 6 months. Hence, these patients have been included in the study only one diabetic patient was included in the study under his specific request.

The patients selected were between 20 to 25 years old (average  $22,4 \pm 2,6$  years). Of these, 45% were female and 55% were males. All patients were in good systemic and periodontal health, as assessed from both their medical history and oral and periodontal examination.

Exclusion criteria for the study were: familiarity with or suspected diabetes, presence of metabolic diseases, use of pharmacological drugs during the month prior to sample collection, presence of gingivitis or periodontal disease, incorrect or unusual nutritional habits, presence of xerostomia or reduced salivary flow.

A single clinician (M.T.) evaluated all patients, using a recent medical history and clinical findings. M.T was also responsible for instructing the patients on correct oral hygiene care and collecting repeated samples. The information recorded was: age, gender, number of teeth and the plaque Index score according to Silness and Loe.

After an additional evaluation, patients received detailed information about the study procedures. All patients have been asked not to brush their teeth, drink or eat within 8 hours prior to the first sample collection (at 8 am). They were also asked to take with

them their toothbrush and toothpaste in order to demonstrate and explain to them the correct oral hygiene techniques.

Salivary samples were always collected between 8 am and 12 pm. Un-stimulated whole saliva from each participant was collected at each study visit via passive drooling into a sterile plastic tube.

The first sample was taken after 8 hours from tooth brushing (from 8:00 am to 8:30 am) or after 90 minutes (following two different Protocols). After the first oral fluid sample collection, patients were asked to eat a standardized breakfast (selected by us). After breakfast, patients were asked to brush their teeth as they would normally. One hour after tooth brushing the second oral fluid sample was collected. Other three samples were taken respectively after 90, 120 and 180 minutes.

One hundred and twenty five total samples were collected and statistically normalized using the Skewness/Kurtosis and Shapiro-Wilk Normality Tests, and then analyzed with the Kruskal Wallis Test and Linear Regression Model. Values where  $p < 0,05$  were considered to be significant.

## Reagents and solutions

Glucose oxidase/oxidase, o-dianisidine dihydrochloride and sodium fluoride were purchased by Aldrich; Sodium azide, D(+)-glucose monohydrate and sulphuric acid (95-97%) were Merck reagents. Artificial saliva was prepared, according to the literature (22) by dissolving in water the following reagents:  $0.9 \text{ gL}^{-1}$  KCl (Aldrich),  $0.66 \text{ gL}^{-1}$   $\text{KH}_2\text{PO}_4$  (Baker),  $0.08 \text{ gL}^{-1}$   $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  (Carlo Erba),  $0.49 \text{ gL}^{-1}$   $\text{KHCO}_3$  (Merck),  $0.37 \text{ gL}^{-1}$   $\text{NaNO}_3$  (Merck),  $0.12 \text{ gL}^{-1}$   $\text{CaCO}_3$  (Merck),  $0.64 \text{ gL}^{-1}$   $\text{NH}_4\text{Cl}$  (Merck),  $0.20 \text{ gL}^{-1}$  Urea (Merck),  $2.00 \text{ gL}^{-1}$  Mucin (Aldrich). All the solutions were prepared with Millipore Milli-Q ultrapure water (resistivity  $> 18 \text{ M}\Omega \text{ cm}^{-1}$ ).

Standard solutions of D(+)-glucose monohydrate were prepared for the calibration plot, diluting a 100 ppm solution.

5 mg of o-dianisidine dihydrochloride were dissolved in 1 mL of water and 0.8 mL of this solution were added to a Glucose oxidase/oxidase solution, prepared dissolving the content of a capsule in 39.2 mL of water.

Solutions of 0.01 M NaF and 0.01 M  $\text{NaN}_3$  were used as glycostatic and bacteriostatic agents respectively, and a 12 N  $\text{H}_2\text{SO}_4$  solution was used to stop the enzymatic reaction.

## Determination method

The method used for the determination of glucose in saliva samples is an optimization of the GAGO-20 Sigma Aldrich Technical Bulletin. It is a spectrophotometric enzymatic technique based on the transformation of glucose to gluconic acid mediated by glucose oxidase and the consequent production of  $\text{H}_2\text{O}_2$ . Hydrogen peroxide reacts with o-dianisidine in the pres-

ence of peroxidase, producing a colored product, whose absorbance, proportional to glucose concentration, is measured at 427 nm.

2 mL of the solution containing glucose oxidase/peroxidase and o-dianisidine were added to each sample to be analyzed; after 30 min at 37° C in a thermostating bath and 2 mL of 12 N H<sub>2</sub>SO<sub>4</sub> were used to stop the enzymatic reaction. The absorbance spectrum was measured in the range 380-800 nm, measuring the maximum at 427 nm.

### Calibration plot

The calibration plot was obtained using calibration solutions gathered by mixing 0.25 mL of 0.01 M NaF, 0.25 mL of 0.01 M NaN<sub>3</sub>, 0.25 mL of artificial saliva and 0.25 mL of standard glucose solutions in the concentration range of 0 to 40 ppm. Each of these samples was treated with the procedure explained before, measuring and correcting the absorbance with the blank value.

### Protocols

Two protocols were established to evaluate the glucose measurements and behavior in human saliva:

- Protocol 1 (12 healthy patients): the first sample was collected after 8 hours from tooth brushing, before breakfast (shown by the red continuous vertical line in Fig. 1) and subsequent morning tooth brushing (shown by the green dashed vertical line in Fig. 1). Saliva was then collected at 60, 90, 120, 180 minutes from breakfast and morning tooth brushing.
- Protocol 2 (9 healthy patients + 1 diabetic patient): the first sample was collected in the morning after 8 hours from tooth brushing, (shown by the green dashed vertical line in Fig. 2). The patients were then asked to have breakfast (shown by the red continuous vertical line in Fig. 2). Saliva was then collected at 60, 90, 120, 180 minutes from breakfast.

For both protocols the standardized breakfast consisted in a sweetened coffee or tea (with 2 teaspoons of sugar corresponding to 8,4 g) and an empty croissant.

The collection procedure of saliva involved taking two samples of 0.5 mL to which 0.25 mL of 0.01 M NaF and 0.25 mL of 0.01 M NaN<sub>3</sub> were added as glycolytic and bacteriostatic agents. The first sample was mixed with 2 mL of the solution containing glucose oxidase/peroxidase and o-dianisidine whilst the second sample was mixed with 2 mL of water (blank). Both samples were treated as described in the method section. The measurements of glucose concentration in the samples were obtained subtracting the blank absorbance and using the calibration plot. The blank subtraction is essential to partially eliminate the contribution to the absorbance caused by the different opacity of the saliva samples, which comes from different patients. In such way, the measurement can only be referred to the glucose content.

### Results

This study involved 21 non-diabetic patients; 11 (52,4%) male and 10 (47,6%) female patients aged between 20 to 25 years. The average age was 22,4 ± 2,6 years. For the whole oral fluid sample the average flow rate was 0,53 ± 0,21 ml/min. A statistically significant correlation between salivary glucose and un-stimulated salivary flow rate was found (P=0,001).

### Calibration plot

The calibration plot (Fig. 2) of the analytical methodology chosen for this study was obtained in the range of 0-4 mg dL<sup>-1</sup> with a positive correlation. The limit of detection was calculated at 0.011 mg dL<sup>-1</sup> of glucose and the limit of quantification at 0.0325 mg dL<sup>-1</sup> of glucose. These results show that the method used is applicable to analyze the glucose content in human saliva.

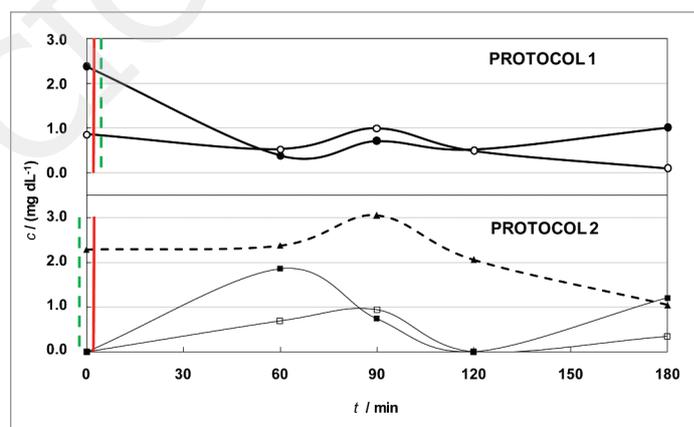


Figure 1. General glucose concentration trend ("pseudo-glycemic curve") for the two Protocols (two examples for each protocol: continuous lines from data in bold in Tables 1 and 2). The dashed line represents the trend of the diabetic patient.

## Discussion

Many authors have proved the efficiency and importance of saliva as diagnostic fluid. Salivary tests to detect both periodontal and systemic illnesses are now an established possibility. In fact, in literature there are plenty of articles available which discuss the applicability of oral fluid sample analyses (from the simplest one to the most complex) as the integrated microfluidic platform for oral diagnostics, usually known as Point-of-care (POC) salivary tests (23-26). Several studies evaluate the concentration of glucose in the saliva of diabetic patients. However, only a minority of them focuses on healthy patients assessed at different times during the day with the purpose of investigating whether their glucose levels vary according to several factors such as tooth-brushing and nutritional habits.

Table 1 and Table 2 show the results of the glucose determination according to both Protocols.

The data collected varies significantly from patient to patient (as expected), probably due to the different metabolism of each individual.

Nevertheless, a particular trend, showing a maximum value around 90 min from breakfast has been observed. Figure 1 shows two examples for each protocol (continuous lines, data in bold in Tab. 1 and 2) representing this trend. This data suggests a correlation between food intake and salivary glucose. However, such correlation has to be further assessed due to the small number of samples analyzed.

The difference between the two Protocols is particularly evident in the first collected samples.

Protocol 1, in which the first sample is collected in the morning before tooth brushing (8 hours after the last tooth brushing), demonstrates that during the night, when the salivary flow rate is reduced, the levels of glucose in oral fluids increases and probably accumulates on oral hard and soft tissues.

Protocol 2, which involves tooth brushing before the first sample collection (precisely 90 minutes before), demonstrates the importance of effective oral hygiene care which reduces the level of glucose in the oral cavity (compared to Protocol 1) up to a measurement of 0 in half of the cases. Moreover, salivary flux activation in these patients, due to tooth brushing, proba-

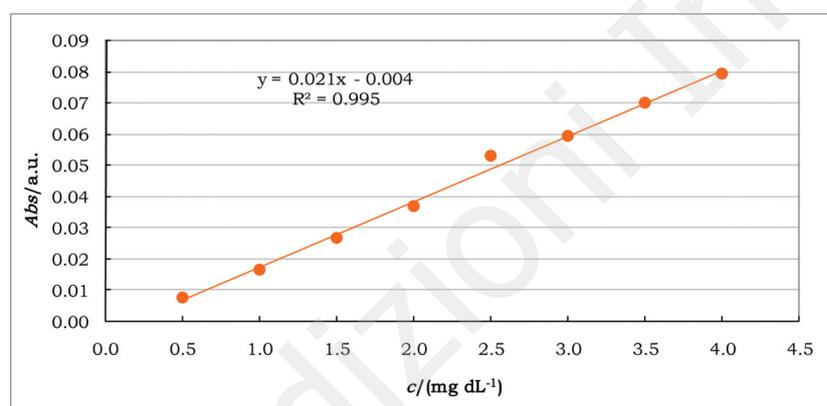


Figure 2. Calibration plot for glucose determination via enzymatic spectrophotometric method.

Table 1. Glucose concentration data for all the samples collected in Protocol 1.

		PROTOCOL 1 PATIENT NUMBER											
Sample	t/min	1	2	3	4	5	6	7	8	9	10	11	12
		$c_{\text{glu}} / (\text{mg dL}^{-1})$											
1	0	<b>2.37</b>	1.10	0.82	0.95	<b>0.85</b>	0.94	0.40	0.62	1.12	1.39	0.68	0.32
2	60	<b>0.38</b>	0.00	0.43	0.61	<b>0.52</b>	0.00	0.00	0.00	0.65	0.09	0.36	0.00
3	90	<b>0.71</b>	0.00	0.66	0.16	<b>0.99</b>	0.30	0.10	0.14	0.14	0.00	0.51	0.65
4	120	<b>0.51</b>	0.00	0.78	0.40	<b>0.49</b>	0.04	0.33	0.07	0.24	0.00	0.82	0.00
5	180	<b>1.00</b>	0.00	0.82	0.46	<b>0.10</b>	0.26	0.35	0.06	0.23	1.32	0.30	0.00

Table 2. Glucose concentration data for all the samples collected in Protocol 2; a diabetic patient has also been recorded.

		PROTOCOL 2 PATIENT NUMBER										
Sample	t/min	1	2	3	4	5	6	7	8	9	Diabetic	
		$c_{\text{glu}} / (\text{mg dL}^{-1})$										
1	0	0.79	<b>0.00</b>	0.42	0.74	0.00	<b>0.00</b>	0.00	0.61	0.28	<b>2.29</b>	
2	60	1.02	<b>1.85</b>	0.72	0.99	0.00	<b>0.68</b>	0.89	0.65	0.18	<b>2.37</b>	
3	90	0.50	<b>0.74</b>	0.92	0.76	0.24	<b>0.93</b>	0.00	0.78	0.73	<b>3.04</b>	
4	120	0.00	<b>0.00</b>	0.55	0.98	1.06	<b>0.00</b>	0.00	0.55	0.32	<b>2.05</b>	
5	180	0.74	<b>1.20</b>	0.77	0.63	0.11	<b>0.33</b>	0.91	0.31	0.00	<b>1.04</b>	

bly plays a role in the salivary glucose concentration observed.

The dashed line in Figure 2 - Protocol 2, shows the behavior of glucose in the diabetic patient. The trend of the "pseudo-glycemic curve" is the same already discussed, but shifted at higher values, demonstrating how it may be possible to use salivary glucose concentration in the monitoring of diabetic patients.

Regarding the concentration of salivary glucose, the average collected from all patients involved in this study was lower than the measurement obtained by Soares et al. (27), which was 5,94 mg/dL and Di Gioia et al. (28) which was 5,57 mg/dL. Agha-hosseini et al. observed an average salivary glucose measurement of 13,6 mg/dL. As proposed by Soares et al. (27), one possible explanation of salivary glucose differences obtained in these studies may be the various study designs as well as the diversity of the methods used and selection criteria of the sample collection (13, 29). This data suggests that domiciliary oral hygiene techniques of each patient should always be assessed before oral fluid sample taking or, if possible, previously set.

In accordance to the results obtained from this study, the majority of authors observe a statistically significant correlation between flow rate and salivary glucose concentration. These suggest a pivotal role of flow rate, not only for the assessment of glucose concentration but also for the identification of biomarkers levels in the diagnosis or screening of systemic and local diseases (27-29).

## Conclusions

Based on the results obtained from the samples studied, it is possible to determine that salivary glucose is present even when plaque levels and oral health status are strictly controlled. However, further studies are required to prove a correlation between glucose intake and salivary glucose as the number of patients and samples collected in our study were limited.

Moreover, an alternative, complementary, more rapid, precise and accurate analytical methodology (capable of following glucose concentration in saliva possibly on-site), is to be designed. In this context, electro analytical techniques, capable of monitoring small quantities of organic compounds in situ (30-31), could be an interesting alternative.

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