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ORIGINAL ARTICLE

Utility of Saliva for Measurement of Thyroid Hormones

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ABSTRACT

Introduction: Saliva as an alternative biological fluid of choice to blood in diagnosing systemic diseases evolved due to its noninvasive nature of collection. There is little information on the levels of thyroid stimulating hormone (TSH, thyrotropin) and T3 levels in saliva. The study was thus taken up to study the suitability of saliva for measurement of thyroid hormones in comparison to serum.

Materials and methods: Fasting saliva and serum samples were collected from 30 healthy individuals for the measurement of total T3 (TT3), total T4 (TT4), TSH, free T3 (FT3), and free T4 (FT4). Timed samples were collected from 10 subjects for the evaluation of diurnal variation.

Results: The thyroid hormones studied, i.e., TT3, TT4, FT4 and TSH were significantly higher in serum when compared to saliva ($p < 0.01$). A significant positive correlation was found between serum and salivary TSH ($r = 0.420$, $p = 0.001$). Variations in salivary TSH reflected the pattern seen in serum TSH. However, there was a lack of agreement between the measurement of TSH in serum and saliva when assessed using Bland Altman and Mountain plots.

Conclusion: Saliva cannot be used as an alternate sample for analysis of thyroid hormones.

Keywords: Saliva, Serum, Thyroid hormones.

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INTRODUCTION

Among the various biological fluids available, saliva as an alternative biological fluid of choice to blood in diagnosing systemic diseases evolved due to its noninvasive nature of collection. Other advantages include ease of collection with more stability at room temperature, less chances of transmission of blood-borne diseases, minimum precautions for storage with minimal or no need of preservatives, and no need of special training for the laboratory personnel to handle saliva sample.[1-3] Its use in monitoring endocrine disorders including Cushing's syndrome, adrenal insufficiency, ovarian function disturbances, testicular diseases, hyperthyroidism, etc.[1,3,4] has been studied.

Studies on hormone measurement in saliva have shown that the salivary hormone levels reflect the plasma free fraction which is the biologically active form of the hormone.[5-8] Previous studies have measured the salivary thyroxine levels using radioimmunoassay (RIA)[9,10] and stable Isotope-dilution liquid chromatography/tandem mass spectrometry (LC/ESI-MS/MS). There is little available information on the levels of TSH, thyrotropin in saliva, which is the most reliable test of clinical importance for diagnosing hypo- and hyperthyroidism in the outpatient setting and follow-up of patients with thyroid dysfunction.[11] Similarly, literature with respect to T3 levels in saliva is limited. T3 testing along with freeT4 (FT4) is useful in diagnosing and monitoring hyperthyroidism.[12,13] The sampling time is important for hormonal assays since the secretion of hormones is under the control of trophic hormones secreted by the hypothalamus.[14] The present study was thus taken up to study the thyroid hormones, i.e., total T3 (TT3), total T4 (TT4), TSH, free T3 (FT3), and FT4 in healthy individuals along with diurnal variations.

MATERIALS AND METHODS

A total of 30 healthy subjects from among the hospital staff and students of Sri Venkateswara Institute of Medical Sciences, Tirupati without any history and evidence of thyroid disease were included after informed consent. Individuals with oral disorders, history of diabetes mellitus, and smoking, acute illness, those on cholesterol-lowering drugs, steroids, and pregnant women were excluded from the study. The study was approved by the institutional ethics committee.

Sample Size Calculation

The sample size was calculated based on previous study[10] using Master statistical software version 2.0, a software developed by Christian Medical College, Vellore.

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Sample Collection

Fasting saliva and serum samples were collected from 30 healthy individuals after informed consent. Timed saliva and serum samples were collected from 10 control subjects for the evaluation of diurnal variation of thyroid hormones. The timings of collection were: in the morning about 7:00 am or just before getting out of bed, between 8:00 and 9:30 am, and around 1:00 pm. Other samples were collected in the evening between 4:00 and 6:00 and before going to bed at 10:00 pm.

About 2 to 3 mL of mixed saliva was collected in graduated tubes, centrifuged, aliquoted, and stored at −80°C until analysis of the samples. A volume of 3 mL of venous blood was collected, allowed to separate serum and separated by centrifugation. Separated serum was stored at −80°C for analysis.

T3, T4, hypersensitive TSH (hTSH), and FT4 were evaluated on Beckman coulter access 2 auto analyzer (USA) by CLIA method and FT3 using DSI kits on Chemwell autoanalyzer ELISA system (USA). The analytical sensitivities of the methods used were 0.1 ng/L (0.2 nmol/L) for total T3, 0.50 µg/dL (6.4 nmol/L) for total T4, 0.25 pg/mL for free T3, 0.25 ng/dL (3.2 pmol/L) for free T4, and 0.01 µIU/mL for hTSH.

Statistical Analysis

Continuous variables were expressed as mean ± SD. Mann–Whitney U-test was used to test the significance of difference in means between serum and salivary parameters studied. The correlation was assessed using Spearman's rank correlation analysis. Agreement between the two estimations was tested using Bland–Altman plot and mountain plot. Data analysis was performed using Microsoft excel spread sheets, SPSS for windows version 11.5 program (SPSS Inc, Chicago, Illinois, USA), and Medcalc statistical software (version 13.2.2, Belgium). A p-value < 0.05 was considered statistically significant.

RESULTS

The mean age of the subjects was 38 ± 12 years. There were 23 (57.5%) males in the present study. All the subjects had normal biochemical profile (Table 1).

The thyroid hormones studied, i.e., TT3, TT4, FT4, and TSH were significantly higher in serum when compared to saliva (p < 0.01). However, no significant differences were found between FT3 in saliva and serum (p = 0.541) (Table 2).

Serum T3 levels showed a significant positive correlation with serum T4 (r = 0.527, p = 0.001). No correlation was found between serum T3 with salivary T3 (r = 0.001). A significant positive correlation was found between serum TSH and salivary TSH (r = 0.420, p = 0.001) (Table 3).

Table 1: Baseline characteristics of the patient population

Parameter	*Mean ± SD (n = 30)*	*Reference range*
Age (years)	42 ± 12	
Plasma glucose (mg/dL)	87.1 ± 5.1	70–110 mg/dL
Serum urea (mg/dL)	23.0 ± 17.7	10–40 mg/dL
Serum creatinine (mg/dL)	0.81 ± 0.16	0.3–1.3 mg/dL
Serum sodium (mmol/L)	139.1 ± 3.4	135–145 mmol/L
Serum potassium (mmol/L)	4.2 ± 0.44	3.5–5.0 mmol/L
Serum total cholesterol (mg/dL)	164.0 ± 38.6	<200 mg/dL
Serum triglycerides (mg/dL)	134.8 ± 46.3	<200 mg/dL
Serum HDL (mg/dL)	40.2 ± 9.4	40–70 mg/dL
Serum LDL (mg/dL)	99.0 ± 34.6	50–140 mg/dL
Serum VLDL (mg/dL)	27.0 ± 9.3	10–40 mg/dL

*Statistically significant; Data are expressed as mean ± SD (standard deviation); HDL: High-density lipoprotein; LDL: Low-density lipoproteins; VLDL: Very low-density lipoprotein

Table 2: Thyroid hormone and TSH levels in serum and saliva in the study group

Parameters	*Serum*	*Saliva*	*p-value*	*Reference range in serum*
TT3 (ng/mL)	1.11 ± 0.16	0.08 ± 0.06	0.001*	0.87–1.78
FT3 (pg/mL)	6.21 ± 1.80	5.93 ± 1.82	0.541	3.6–6.0
TT4 (ng/mL)	95.63 ± 13.36	4.49 ± 1.46	0.001*	78.38–157.4
FT4 (ng/mL)	0.91 ± 0.12	0.13 ± 0.11	0.001*	0.61–1.12
TSH (µIU/mL)	2.29 ± 1.63	0.31 ± 0.62	0.001*	0.34–5.60

*Statistically significant; Data are expressed as Mean ± SD (standard deviation); TT3: Total triiodothyronine; FT3: Free triiodothyronine; TT4: Total thyroxine; FT4: Free thyroxine

The diurnal variation in thyroid hormones was studied by collecting blood and saliva samples from 10 individuals at multiple time points during the day. Serum Total T3 levels showed a fall around 1.00 pm and the levels increased toward night. However, serum free T3 remained relatively constant during the day with a slight fall during the night. Serum total T4 and serum free T4 increased toward evening and a fall during night. The levels showed a 30% fall between 8.00 am and 10.00 am. Similarly, serum TSH levels increased from afternoon with maximum levels at night (10.00 pm).

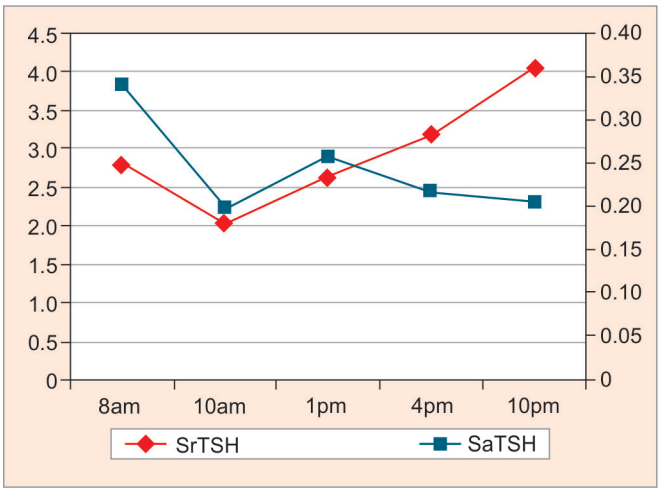
With respect to saliva, salivary TSH levels reflected the pattern seen in serum TSH with the levels showing a fall between 8.00 am and 10.00 am. The levels remained relatively constant during other times of the day (Graph 1). Salivary T3 levels showed a rise from morning till 1.00 pm. Salivary free T3 levels remained constant during all times. Salivary T4 levels and salivary free T4 levels showed a rise from morning till 1.00 pm, after which the levels decreased.

Since a positive correlation was found between serum and salivary TSH measurements, the two were tested for

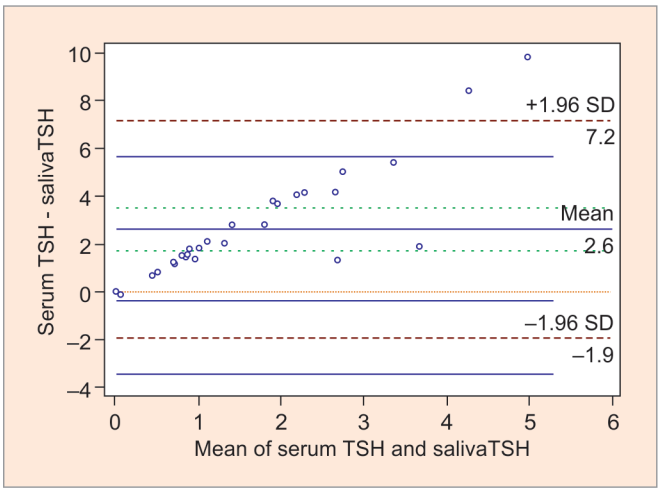
Table 3: Correlation matrix between thyroid hormones in saliva and serum

	sa_TT3 r	*sa_TT4 r*	*sa_TSH r*	*sa_FT3 r*	*sa_FT4 r*	*sr_TT3 r*	*sr_TT4 r*	*sr_TSH r*	*sr_FT3 r*	*sr_FT4 r*
sa_TT3	1	0.075	−0.176	−0.322	−0.245	0.197	0.046	−0.368	−0.189	−0.108
sa_TT4		1	−0.205	0.016	0.122	−0.262	−0.085	0.083	0.049	−0.393*
sa_TSH			1	0.340	−0.011	0.267	0.315	0.420*	−0.014	−0.077
sa_FT3				1	−0.307	0.055	0.169	0.056	0.136	0.123
sa_FT4					1	−0.035	0.059	−0.036	−0.033	0.042
sr_TT3						1	0.527**	0.042	0.085	−0.020
sr_TT4							1	0.240	0.269	0.019
sr_TSH								1	0.182	−0.051
sr_FT3									1	0.150
sr_FT4										1

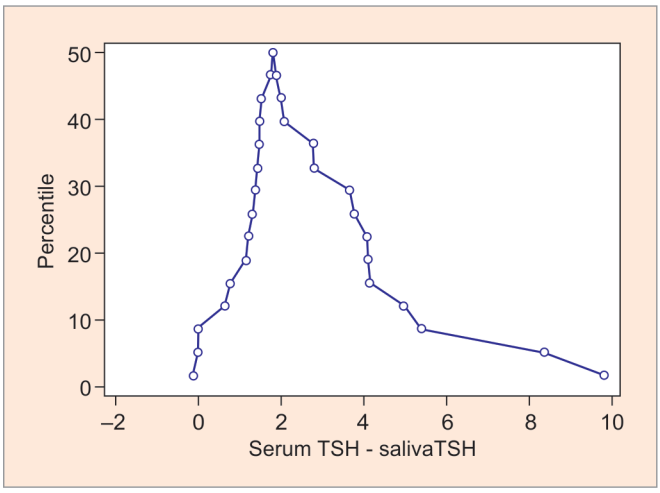
sa: Salivary; sr: Serum; r: Correlation coefficient; T3: Triiodothyronine; T4: Thyroxine; FT3: Free triiodothyronine; FT4: Free thyroxine; *Correlation is significant at the 0.05 level (2-tailed); **Correlation is significant at the 0.01 level (2-tailed)



Graph 1: Diurnal variations in serum and salivary TSH (n = 10)



Graph 2: Bland Altman plot of serum TSH compared to salivary TSH



Graph 3: Mountain plot for serum TSH and salivary TSH

agreement using Bland Altman and mountain plots. There was lack of agreement in the measurements of TSH in serum and saliva as shown in Graphs 2 and 3.

DISCUSSION

Among the laboratory investigations, estimations of TT_3, TT_4, and TSH concentrations in blood represent the functional status of thyroid gland and are routinely used in clinical practice. Due to its noninvasive nature and ease of collection, the utility of saliva as a diagnostic fluid for measuring salivary thyroid hormones was explored in the present study.

Concentration of thyroid hormones TT3, TT4, and TSH levels were significantly higher in serum when compared to saliva. The serum TT4 levels were 25 times higher than saliva TT4 levels. Similarly, TT3 and TSH levels were significantly higher in serum compared to saliva ($p = 0.000$). A significant positive correlation was seen between serum and salivary TSH ($r = 0.420$).

It has been proposed that salivary hormones represent the free fraction of the hormone.[7,8] The concentrations of free T_4 and free T_3 were estimated both in serum and in saliva in the present study. Serum FT_4 levels were significantly higher than salivary FT_4 (Table 2). Serum FT4 levels were found to be tenfold higher than saliva fT_4 but five fold lower than saliva TT4. The difference observed between the fT4 concentration in saliva and that of serum free T4 has been proposed to be due to active transport of T4 from the extracellular fluid and its concentration in saliva by the salivary gland and also the fact that some T4 is transported into saliva bound on albumin which is present in saliva in minute concentration[9] Higashi et al,[15] using a

stable isotope-dilution liquid chromatography/tandem mass spectrometry method, demonstrated a diagnosable difference in T4 levels in euthyroid subjects compared to patients with Graves disease. Using a simple radioimmunoassay, Putz et al,[10] also reported good agreement between salivary T4 levels and the functionality of thyroid.

The measurement of T_4 in saliva as an index of free T_4 in plasma is said to be complicated by the presence of T4-binding proteins in saliva, mainly albumin.[9] Also, conditions with abnormal levels of thyroid binding globulins (TBG) as seen in pregnancy, in congenital deficiency of serum TBG, can pose a problem during analysis. Contamination of saliva with plasma or gingival fluid is likely to cause a large variability in the concentration of T4 in saliva due to the very large total-to-free ratio for T_4 in plasma 5000:1.[16]

Hence, it was proposed that measurements of the T4 concentrations in whole saliva are unlikely to give an accurate index of the plasma concentrations of free T4.[16] Salivary albumin levels were measured in order to assess any contamination with plasma in the present study. However, the levels were undetectable, ruling out contamination with plasma. Another probable cause can be metabolism of T4 in saliva to T3 by iodothyronine 5′-deiodinase. Sublingual glands have been shown to have iodothyronine 5′-deiodinase activity in experimental studies.[17] Sublingual 5′-deiodinase activity has been shown to be approximately 80% of that in the liver. This theory is supported by the findings of salivary and serum FT3 levels in the present study. Salivary and serum FT3 levels were similar in the present study ($p = 0.541$). The net salivary hormone levels can be said to be due to passive diffusion and ultrafiltration of the free fraction and the peripheral metabolism of the hormones in the salivary glands.

Thyroid hormone levels in serum are under control of TSH. The measurement of serum TSH levels reflects the integrity of the hypothalamic–pituitary–thyroid axis. In general, serum and salivary levels of protein hormones are not well correlated. These hormones are too large to reach saliva by means of passive diffusion across cells or by ultrafiltration, and the detection of these hormones in saliva is primarily due to contamination from serum through gingival crevicular fluid or oral wounds. Therefore, serum levels of protein hormones, such as gonadotrophins, prolactin, and thyrotropin cannot be accurately monitored by means of salivary analysis.[16] Though a significant positive correlation was observed between salivary and serum TSH levels ($r = 0.420$, $p < 0.05$), salivary levels do not accurately reflect the serum levels as seen from the scatter plots and the plots of agreement. The correlation coefficient "r" depends on the between-subject variability which increases as the between-subject variability increases. Correlation coefficient is not a measure of agreement, but a measure of association[18] and hence a high correlation does not infer that the methods can be used interchangeably.[19]

To assess the agreement between the two methods, the first approach is visualization of scatter plots using the two methods. Observations should cluster along the regression line. This again depends on the range of measurements. As the range of measurements increases, the better is the agreement. Bland Altman plot is a powerful way of displaying the results of difference in measurement (A – B) against (A + B)/2 the average.[18] As shown in Graph 2, there is a proportional error between the measurements of TSH in serum and saliva. Also the mountain plot shows the differences to be centered away from zero, showing lack of agreement between the methods (Graph 3).

We also looked into the diurnal variations in salivary thyroid hormone levels. Many hormones exhibit circadian and diurnal variations. There is evidence for diurnal variation in serum TSH levels, with a maximum around midnight.[20] Similarly, in the present study, serum TSH levels were found to increase from afternoon with maximum levels at night (10.00 pm). The levels showed a 30% fall between 8.00 am and 10.00 am. Similarly, serum total T3 levels showed a fall around 1.00 pm and levels increased toward night. However, serum free T3 remained relatively constant during the day with a slight fall during the night. Serum total T4 and serum free T4 increased toward evening and a fall during night.

With respect to saliva, salivary TSH levels reflected the pattern seen in serum TSH with the levels showing a fall between 8.00 am and 10.00 am. The levels remained relatively constant during other times of the day (Graph 3). Salivary T3 levels showed a rise from morning till 1.00 pm. Salivary free T3 levels remained constant during all times. Salivary T4 levels and salivary free T4 levels showed a rise from morning till 1.00 pm after which the levels decreased. The circadian rhythm has also been reported. A pulsatile release of hormones from the thyroid gland governed by a pulsatile TSH secretion has been shown to account for 13, 15, and 11% of the mean level of TSH, free T3 and free T4 respectively.[20] Therefore, timing of saliva may also affect the results.[21]

Elson et al[9] reported a spike in salivary T4 levels (2–3 times basal) in one subject sampled repeatedly throughout the day. However, Higashi et al[15] suggested that the sample collection time does not affect the salivary T4 testing as a good agreement was found in five euthyroid subjects sampled at 10 am and 6 pm variation with ± 20% variation in their study.

CONCLUSION

The findings of the present study show that though salivary TSH levels showed a positive correlation with serum TSH levels, it cannot be used instead of serum TSH levels for patient management as evident from the poor agreement and the Bland Altman plots. Thus, saliva is not the sample of choice for analysis of thyroid hormones.

LIMITATIONS

The sample size was small and also analysis of thyroid hormones in saliva was not done in patients with thyroid disorders. This was planned only if a positive result would have been obtained in euthyroid individuals.

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