Derivatised liquid chromatographic method for statistical estimation of saliva samples by non-invasive technique

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INTRODUCTION

Diabetes mellitus (Pohl et al., 1985) is a group of metabolic disorders that share the common underlying feature of hyperglycemia. Hyperglycemia in diabetes results from defects in insulin secretion, insulin action, or, most commonly, both (Omar and Khalil, 1999; Amer et al., 2001).

The blood glucose level in diabetes patients determines by an invasive procedure (Ginsberg, 1992; Chiappin et al., 2007). Whereas, the non-invasive method is fast, painless, cost-effective. This primary concern in non-invasive (N.I.) glucose measurement...
in saliva samples achieved high accuracy, and no blood is involved in the process (Kaufman and Lamster, 2002).

Subsequently, the general study, to quantitatively estimate the amount of salivary glucose (Mehrotra et al., 1968a) levels in kind-2 diabetic patients and discover the possibility of the use of saliva to mirror the glucose levels in the blood, thereby making self-measurement of Blood-glucose less invasive (Mehrotra et al., 1968b). Based on the literature survey there was limited analytical and statistical methods for available. There was a lack of a sensitive analytical method for the identification and quantification in saliva samples. And there was no sensitive analytical method detect to quantify in saliva samples by HPLC (Marchetti et al., 1989; Huizinga and Rothman, 2006).

MATERIALS AND METHODS

Instrumentation

H.P.L.C., pump and U.V. detector (Agilent 1200), H.P.L.C., PABA AR grade, Sodium Acetate (A.R. Loba Chemie), Acetonitrile (H.P.L.C.), Water H.P.L.C., Diethyl Ether AR (Merck).

Method optimisation and Sample Cleanup

After series of trials, the chromatographic conditions were accomplished with the buffer having the 50mM Sodium acetate and acetonitrile (60:40% v/v) by utilising the stationary phase Phenomax-C18 (250 x 4.6 mm, 3.5m) to obtain the best peak shape and separation was good at 254 nm with the column temperature 25°C and sample compartment temperature 10°C with the flow 1.0 ml/min with the sample volume 20μl and eluted at 3.4±0.02 min.

To each labelled polypropylene tube, 2.5 mL of diethyl ether was added containing 100 μL of a saliva sample and vortexed for 15 min. The samples were centrifuged at 4000 rpm for 15 min at ambient temperature. The supernatant phase was transferred to a clean P.P. tube and dried with nitrogen gas at 40°C up to 20 min. The residue was reconstituted with 0.2ml of 0.2% w/v PABA reagent and 500 μL mobile phase. The mixture was heated at 70 °C for 30 min, cooled the sample and 20 μL sample was injected into HPLC.

System suitability

To verify the system producing the consistent results with the optimised method injected the standard for six times with the criteria of % R.S.D. for retention time and area NMT 2.0%, theoretical plates NLT 3000 plates, tailing factor NMT 1.5 and resolution N.L.T. 4.

Selectivity and Specificity

To verify the method validation in terms of the selectivity and exactness injected triplicate preparations of 100 μg/ml. Then injected one blank also to prove the method was not having the carryover issue. The limit for the specificity is, it should pass the system suitability criteria, and there should not be R.T. shift for all the three preparations. The blank chromatogram was depicted in Figures 1 and 2.

Linearity

The method linearity was verified with the five concentrations of 40.00, 60.00, 80.00, 100.00, 200.00, 400.00, 600.00 μg/mL acceptance criteria of the regression coefficient (R²) NLT 0.99.

Sample collection and preparation

Salivary sample collections were collected in the clinical laboratory in the early morning on 6-7 am with an empty stomach. After 2 hours of breakfast, subjects were asked to wash their mouths with distilled water and to spit two or three times in wash sink, after which they were told to spit the saliva pooled in their mouths for the following 5 minutes into the sterile sample collection container saliva and samples were centrifuged. Glucose was estimated in the supernatant saliva by the glucose pre-column derivative RP-HPLC method. Statistical analysis of the obtained data was conducted using O.R.I.G.I.O.N. statistical software version 2007.1. Means and standard deviations (S.D.s) were calculated for the individual groups. These were then compared using independent Student’s t-test, Fisher’s test and regression coefficient.

RESULTS AND DISCUSSION

The developed approach indicates less matrix effect at analyte retention time. The chromatograms of clean and saliva pattern were depicted in Fig-1&2.

Figure 1: Chromatogram of Blank Matrix (Saliva)

Comparisons of salivary glucose levels between the control and patient groups were performed with a t-test. There is high statistical significance between the serum blood glucose level and salivary glucose level. A distinct difference was observed in the
Table 1: Statistical evaluation of Serum & salivary Blood-glucose levels in non-diabetic subjects (Control)

| Glucose level in | Control (Non-Diabetic) |  |  |
|------------------|------------------------|---|---|
|                  | Fasting (m.g./d.l)     | Post-prandial (m.g./d.l) | t-Value (P<0.05) |
| Serum            | 80.41-87.67            | 99.79-106.8                | *25.64          |
| Saliva           | 1.1-2.99               | 4.63-6.87                  | *39.39          |
| f-Value (P<0.05) | **23.42                | **45.68                    | -               |

* t-value shows a statistically significant difference between serum glucose and salivary glucose.
** f-value shows a statistically significant difference between serum glucose and salivary glucose.

Table 2: Statistical evaluation of Serum & salivary Blood-glucose levels in Diabetic subjects

| Glucose level | Test (Diabetic) |  |  |
|---------------|----------------|---|---|
|               | Fasting (m.g./d.l) | Post-prandial (m.g./d.l) | t-Value (P<0.05) |
| Mean of Serum | 128.32-151.97    | 195.65-217.77             | 57.38           |
| Mean of Saliva| 4.63-6.87       | 10.57-16.19               | 23.56           |
| f-Value (P<0.05) | 57.63            | 25.87                      | -               |

* t-value shows a statistically significant difference between serum glucose and salivary glucose.
** f-value shows a statistically significant difference between serum glucose and salivary glucose.

In the control group during fasting and postprandial state, the mean blood Serum glucose levels ranged from 80.41 to 87.67 mg/dl, 99.79 to 106.67 mg/dl and mean salivary glucose levels ranged from 1.10 to 2.99 mg/dl, 4.63 to 6.87 mg/dl. The correlation coefficient between serum glucose and salivary glucose was calculated, and the r-value was found to be 0.931 and 0.990, which was statistically significant (P<0.05). The results were depicted in Figures 3 and 4 and Table 1.

In the diabetic group during fasting and postprandial state the mean Serum glucose levels ranged from 128.32 to 151.97 mg/dl, 195.65 to 217.77 mg/dl and mean salivary glucose levels ranged from 4.63 to 6.87 mg/dl, 10.57 to 16.19 mg/dl. The correlation coefficient between serum glucose and sali-
Figure 6: Correlation of P.S.G. and P.P.G. levels in diabetic

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vary glucose was calculated, and the r-value was found to be 0.915 and 0.961, which was highly significant (P<0.05). It is worth noting that the significance of the study group was much greater than that of the control group. The results were depicted in Figures 5 and 6 and Table 2.

Comparisons of salivary glucose levels between the control and patient groups were performed with an independent t-test, and the difference was highly significant (P< 0.05), as shown in Tables 1 and 2. The preprandial salivary glucose levels between the control and patient groups were compared using an f-test, and the difference was found to be highly significant (P< 0.05). No significant differences were found (P>0.05) in comparing salivary glucose levels across different patients. Glucose was detectable in saliva of both diabetic and non-diabetic individuals. A significant positive correlation was established between blood glucose and salivary glucose levels. Values observed regarding blood and saliva glucose level were found distinctly different between normal and diabetic subjects suggesting that monitoring of saliva glucose level can be used as an index of diabetes mellitus. The results were depicted in Figures 5 and 6.

CONCLUSIONS

From the present study, it was observed that the best cut off value for fasting salivary sugar level was 2.0 mg/dl and postprandial salivary glucose level was 4.0 mg/dl for patients. These estimated values could be taken as reference values, and similar studies should be done at different states or regional centres to validate a standard salivary glucose level to be used for screening of diabetes as a non-invasive, cost-effective and straightforward method.

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Conflict of Interest

The authors declare that they have no conflict of interest for this study.

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