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Journal of Chromatography B, 801 (2004) 355–358
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Short communication

Quantitative analysis of human salivary glucose by gas chromatography–mass spectrometry

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Received 13 October 2003; received in revised form 14 November 2003; accepted 17 November 2003

Abstract

A reference analytic methodology was developed for the determination of human salivary glucose concentration. The technique involves the glucose derivatization with acetic anhydride and subsequent analysis of glucose penta-acetylated by gas chromatography combined with mass spectrometry. Glucose concentration in the biological fluid depends on the physiological status of the donor.

Keyword: Glucose

1. Introduction

The enhanced interest of researchers towards saliva as a diagnostic fluid [1,2] is clearly due to the high availability of this complex physiological fluid containing proteins, enzymes, carbohydrates, hormones [3]. The concentration level of one and/or some of these components could be associated to particular diseases. In particular, high concentration level of salivary glucose is associated to diabetes disease even if some experimental results deny this assumption [4]. In analogy, contradictory opinion is reported on the correlation between concentration of glucose in saliva and in blood [5]. The analytical procedure available to determine the concentration of salivary glucose is based on sugar reducing capacity and is characterized by a low specificity and sensitivity [6]. Methodologies involving enzymatic reactions that utilize hexokinase or glucose oxidase are useful in the evaluation of glucose concentration in whole saliva. Although with these methodologies the sensitivity is increased to a lower limit of approximately 10 μmol/l, accuracy is still low [7].

High-performance ion-exchange chromatography (HPIEC) combined with pulsed amperometric detection (PAD) has been used to detect salivary glucose with high reproducibility and sensitivity [8]. Separation of carbohydrates, by ion-exchange chromatography requires basic pH and under typical conditions for PAD detection, glucose could co-elute with unidentified [7].

Aim of this work is the development of a sensitive and accurate analytical method to measure salivary glucose concentration. Gas chromatography combined with mass spectrometry (GC/MS), operating in single ion monitoring (SIM) conditions, has been chosen as analytical technique to detect and quantify salivary glucose.

2. Experimental

2.1. Chemicals

D-Glucose-6,6-d_2, glucose and acetic anhydride were purchased from Sigma–Aldrich. Pentacetyl-D-glucose was prepared with excellent purity following a standard procedure [9].

2.2. Saliva samples

Unstimulated whole saliva was collected into vials (5 ml) on ice. Four healthy donors, all dentate participated in this study. After sample collection, 0.15 ml of internal standard

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doi:10.1016/j.jchromb.2003.11.024

(stock A) and 5 mg of NaF were added to 1.5 ml of saliva. After adding 1 ml of $CHCl_3/CH_3OH$ solution, the samples were clarified by centrifugation at 3000 rpm for 10 min at 4 °C. The solution was transferred to another test-tube and the resulting solid was washed with water (1 ml). The combined aqueous layers were dehydrated at 30 °C. To the dried samples were added $CHCl_3$ (1 ml), a catalytic amount of $ZnCl_2$ and $(CH_3CO)_2O$ (0.8 ml). The resulting mixture was stirred for 1 h at 50 °C. Then, the reaction mixture was basified with an aqueous solution of Na_2CO_3 (pH 9) and extracted with 3 ml of $CHCl_3$. $CHCl_3$ extraction was repeated twice. The combined organic layers were evaporated at 30 °C and the resulting residue was dissolved in 1 ml of $CHCl_3$. An aliquot (1 μl) of the obtained solution was injected into GC/MS system. Pentacetylated-D-glucose and the internal standard penta-acetylated-D-glucose-6,6-d_2 were detected by SIM methodology monitoring the ions *m/z* 200 and *m/z* 202, respectively.

2.3. Preparation of standard solutions

Two standard stock solutions of 500 ppm of D-glucose (stock B) and per-acetylated-D-glucose (stock C), and two internal standard stock solutions of 500 ppm of D-glucose-6,6-d_2 (stock A) and penta-acetylated-D-glucose-6,6-d_2 (stock D) were prepared in double distilled water. Two concentration levels of glucose were prepared using stocks B and A: mixture **1a** (50 ppm of internal standard and 25 ppm of D-glucose) and mixture **1b** (100 ppm of internal standard and 250 ppm of D-glucose).

2.4. Calibration standards

Ten concentration levels of penta-acetylated-D-glucose were prepared using stocks C and D: mixture **1c**–**5c** (50 ppm of penta-acetylated internal standard and 10, 20, 40, 80 and 120 ppm of penta-acetylated-D-glucose, respectively) and mixture **1d**–**5d** (100 ppm of penta-acetylated internal standard and 50, 100, 200, 300 and 400 ppm of penta-acetylated-D-glucose, respectively). Calibration standards (**1c**–**5c**) and (**1d**–**5d**) were analyzed by GC/MS and the peak area ratios were plotted against theoretical concentration ratios. Calibration curves for each series were obtained from weighted least-square linear regression analysis of data [$y = (0.028 \pm 0.001)x + (0.018 \pm 0.049)$, $R^2 = 0.993 \pm 0.008$; $y = (0.016 \pm 0.007)x + (0.571 \pm 0.058)$, $R^2 = 0.998 \pm 0.006$].

2.5. GC/MS conditions

GC/MS analyses were performed on a Hewlett-Packard HP5972 A linked to a HP5890 A series II gas chromatograph (Hewlett-Packard), equipped with a HP-5Ms (30 m × 0.25 mm, PhMesiloxane 5%) capillary column. The mass spectrometer was operated at 1000 resolution in electron impact ionization mode [EI (+), 70 eV] with a source temperature set at 200 °C and scanned from 35 to 450 uma at the scan speed decade/1 s, with 0.2 s inter-scan time. Quantitative GC/MS analysis was carried out detecting the target compounds by single ion monitoring. Identification of the organic compounds was performed comparing the EI spectra and chromatographic retention times with those of commercially available authentic reference compounds. Operating conditions: carrier gas: helium, constant flow; split flow: 50 ml/min; column flow:1 ml/min; splitless time: 1 min; injector temperature: 250 °C. Temperature programs: analysis procedure: 60 °C for 2 min, 14 °C/min to 280 °C, held for 20 min. One microliter of sample solution is injected.

Table 1
Concentration (mg/l) levels of: (a) glucose of whole saliva; (b) salivary glucose (a) + 20 ppm of glucose; (c) salivary glucose (a) + 40 ppm of glucose

	Saliva (a)	Saliva (b)	S.D.	*V*	Saliva (c)	S.D.	*V*
Intra-day	19.3	38.3	0.71	0.51	58	0.83	0.7
	83.8	102.1	0.84	0.71	121.8	0.79	0.65
Inter-day	19.3	38.2	0.75	0.57	57.8	0.93	0.86
	83.8	101.9	0.94	0.9	122.2	0.92	0.86

S.D. and *V* are standard deviation and coefficient of variation of the observed concentration.

2.6. Validation

Two milliliters of each mixture (**1a**) and (**1b**) were treated with $(CH_3CO)_2O/ZnCl_2$ as above described. The organic extracts for each concentration level were analyzed and calculated against the calibration curve. To test the repeatability and the reproducibility of the analyses, the same derivatized glucose samples (25 and 250 ppm) were injected five times during the same day (medium value (MV) = 24.60 and 246.2, respectively; standard deviation (S.D.) = 0.76 and 0.72, respectively; coefficient of variation (*V*) = 0.58 and 0.51, respectively) of the observed concentration and for 5 different days (MV = 24.30 and 245.0, respectively; S.D. = 0.64 and 0.90, respectively; *V* = 0.40 and 0.81, respectively). The intra- and inter-day repeatability and stability were also determined by analyzing derivatized whole saliva, previously additioned, of glucose (Table 1).

3. Results and discussion

The analytical technique (GC/MS) selected to determine salivary glucose concentration requires the derivatization of glucose in such a way to increase its volatility and thermal stability. This is accomplished by esterification of all hydroxyl functions with acetic anhydride [8]. The aqueous matrix of the samples associated to the low solubility of glucose in the commonly used organic solvents prevent the direct esterification of the analyte. Measure of salivary glucose clearly depends also on the sample pretreatment adopted.

Table 2
Concentration (mg/l) level of salivary glucose before food ingestion

Day	Donors			
	A	B	C	D
1	18.09	83.68	18.77	24.30
2	16.88	42.06	17.90	15.76
3	9.09	54.02	15.16	41.88
4	18.54	58.03	22.66	42.06
5		49.63		
6		47.08		

Hence, the development of a simple and fast pretreatment procedure is essential to obtain the complete derivatization of glucose and consequently a high recovery. Therefore an accurate analysis requires the inhibition of glucose metabolism by glycolytic enzymes; the efficiency of sodium fluoride to restrain their activity [8] is exploited in the first step of the preparation of the sample. The second step consists in addition a $CHCl_3/CH_3OH$ solution, which allows the precipitation of macromolecules such as proteins and enzymes. The subsequent centrifugation affords an aqueous solution rich in hydrophilic compounds. The aqueous solution is dehydrated at 30 °C and transformed in the corresponding penta-acetylated derivatives. This is accomplished by the addition of catalytic amounts of zinc chloride and acetic anhydride; acetic anhydride is at the same time reagent and solvent. D-Glucose-6,6-d_2 was designated as the optimal internal standard. Pentacetylated-D-glucose and the internal standard penta-acetylated-D-glucose-6,6-d_2 are detected by SIM methodology monitoring the ions *m/z* 200 and *m/z* 202, respectively. The daily glucose concentration measured before food ingestion is showed in Table 2. The glucose level of different donors comes within 9.09 and 83.68 mg/l. In particular, concentration level of glucose for normal healthy donor B in 6 different days (Table 2) is within 42.06 and 83.68 mg/l (MV = 55.75 mg/l).

Additional experiments performed collecting saliva samples on awakening (Table 3) show that this concentration level, for each donor, is clearly higher than the concentration measured during the day. In fact the donor B on awakening shows glucose values within 91.01 and 132.65 mg/l (Table 3) while before food ingestion (5 h after wake up) the concentration level of glucose is within 42.60 and 83.68 mg/l.

Table 3
Concentration (mg/l) level of salivary glucose on awakening

Day	Donors		
	A	B	C
1	225.07	100.62	151.21
2	131.88	95.23	103.47
3	133.23	91.01	169.63
4		111.34	221.75
5		111.64	
6		132.65	

Table 4
Concentration (mg/l) level of salivary glucose of donor B on awakening and after 3 h

Donor	Day	On awakening	3 h after wake-up
B	1	127.25	64.96
	2	121.62	63.84
	3	132.41	64.32
	4	137.00	62.09

To confirm and to correlate the obtained data, two saliva samples of donor B collected on awakening and after 3 h for 4 days are analyzed according the above described procedure (Table 4). The daily medium levels of glucose determined on awakening and 3 h after wake up (MV = 129.57 and 63.80, respectively) suggest that this concentration is probably correlated to the physiological activity of the donor.

In particular, the higher concentration of salivary glucose on awakening is probably due to a greater production of saliva during the night by sublingual gland, which contains both serous- and mucin-secreting cells. Saliva secreted by this glands is viscous and poor of water. After wake up, the secretion of parotid and submandibular glands is activated and the viscosity of saliva usually decreases, hence, the concentration level of glucose declines. Therefore, the composition of saliva changes during the day. The variation of salivary glucose was also measured in different hours of the same day in saliva samples of the same donor B (Table 5).

The higher glucose concentration level determined on awakening could be ascribed to the nightly salivary debit [10]. Several factors, such as chewing-gum mastication, use of citric acid, stress etc afford to a salivary debt which could reflect in higher concentration of glucose. The salivary secretion (volume versus unit of time) could be affected by several factors therefore is not easy to establish a variation factor useful to relate measured glucose concentration in saliva to the blood glucose concentration [8,11]. The glucose concentration depends also on the salivary flow therefore the collection of saliva samples should be standardized for a possible use in diagnostic field.

Table 5
Concentration level of salivary glucose within the 24 h

Donor B	
Hours	Concentration (mg/l)
7:30	137.0
13:50	60.9
15:30	38.3
17:30	42.7
21:00	77.0
23:00	68.7
7:30	171.6

4. Conclusions

The analytical method developed in this study for the determination of salivary glucose concentration is sensitive, accurate and requires a simple preparation of the samples. In particular, GC/MS/SIM methodology, involving the use of the penta-acetylated-D-glucose-6,6-d_2 as internal standard, allows to evaluate concentration up to 20 μmol/l of penta-acetylated glucose. The obtained data show that the glucose concentration on awakening is very high and depends on salivary flow.

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