METHODS

Assessment of Biochemical Characteristics of the Saliva Using Fourier Transform Mid-Infrared Spectroscopy

S. A. Khaustova, M. U. Shkurnikov, E. S. Grebenyuk, V. G. Artyushenko, and A. G. Tonevitsky

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 148, No. 11, pp. 597-600, November, 2009
Original article submitted September 11, 2009

We developed a method of rapid assay for biochemical properties of the saliva based on attenuated total reflection infrared spectroscopy. This method allows evaluating saliva composition within 5 min without sample preparation; 10 μl sample is enough for the analysis. The concentration of total protein, glucose, secretory immunoglobulin A, urea, amylase, cortisol, inorganic phosphate in the saliva can also be measured. Precision and reproducibility of the evaluated parameters are comparable to those obtained by routine clinical analysis.

Key Words: *saliva; infrared spectroscopy; attenuated total reflection infrared spectroscopy; non-invasive diagnostics*

Human saliva is a unique subject for investigation and diagnostics. Association of physiological illness with physiological activity of the salivary glands suggests the possibility of using the saliva as the source of diagnostic information, which possesses a number of advantages over analysis of other biological fluids [1,12]. Various serum or urine components can also be revealed in the saliva [1,2,10,12]. Drug or hormone concentrations in the saliva do not depend on saliva flow rate and sometimes correlate with blood concentration of these substances [7,9,10]. A large number of biomarkers is measured in the saliva, including heavy metals (*e.g.* lead) [6], hormones (cortisol, testosterone) [7,9], toxins and their metabolites (cotinine) [8], enzymes (lysozyme, α-amylase) [13], immunoglobulins (IgA) [9,13], proteins and DNA. Detection and quantitative analysis of biochemical characteristics of the saliva in mid-infrared (IR) region (4000-700 cm^{-1}) are not used in the diagnostics, although its components possess highly specific bands in this region at a particular set of wave numbers depending on the molecular composition and structure [4,11]. The use of mid-IR spectroscopy in quantitative analysis of biological fluids is known for a quite a long time. Glucose, albumin, triglycerides, total protein and immunoglobulin content in blood serum and urine samples can be precisely determined [3,5,14]. Recently, non-invasive methods of diagnostics and estimation of the functional state of the organism, based on the analysis of saliva composition attracted much attention. The development of a method which would combine high reproducibility, precision and simplicity of quantitative assessment of saliva biochemical characteristics is an interesting problem.

The aim of the study was to develop a method of evaluation of biochemical characteristics of the saliva using attenuated total reflection (ATR) infrared spectroscopy and mathematic processing, based on the method of projection on latent structures. The method should be highly precious and reproducible, should differ from traditional biochemical methods by the

Institute of Physical Culture and Athletics, Moscow, Russia. ***Address for correspondence:*** svetakhaustova@gmail.com. S. A. Khaustova

0007-4888/09/1485-0841

absence of sample preparation procedures, and should provide instantaneous information on the concentration of biochemical substances in the studied saliva sample (sample volume ≤10 μl).

MATERIALS AND METHODS

The study was conducted on 28 volunteers. The experiment was approved by ethical committee of All-Russian Research Institute of Physical Culture and Sports. Saliva samples were collected using Salivette pads (Sarstedt). The examinee chewed a cotton pad for 3 min, then the pad was placed into a container with a plastic top. The container was put in a plastic test tube; the saliva was collected on the bottom of the tube by centrifugation at 3000 rpm at 20°C for 20 min. Each sample was divided into 2 parts: one was used for traditional biochemical analysis and the other was used for Fourier transform IR spectroscopy. The samples were kept at -80°C.

IR spectra of samples were recorded using Microlab spectrometer (A2 Technologies in 4000-700 cm^{-1} region) fitted with an ATR attachment (Fig. 1) providing triple reflection of the IR beam penetrating into the analyzed sample to a specific depth. The volume of saliva sample was 2 μl. The sample was dried on the spectrometer attachment for 3 min and the spectrum of the obtained film was recorded. Air spectrum was used as the background. Background and sample spectra were taken with resolution of 4 cm^{-1} and number of scans of 32. Before the analysis of each sample, the attachment was cleaned with distilled water. The analysis was repeated 10 times for each sample, the obtained spectra were averaged and used for calibration plotting and further analysis.

Detection of total protein concentration in the saliva was performed using Coomassie Plus (Bradford) Protein Assay (Piece). Total sIgA level in the saliva was detected using Secretory IgA test system (Vector-Best). Cortisol concentration in the saliva was detected using commercial Salivary Cortisol test system (DRG). Biochemical analysis of glucose, α-amylase, urea, non-organic phosphate was performed using automatic biochemical analyzer HumaStar 300 (Human) and a set of necessary reagents of this company. Fourier-transform IR spectrometry was performed using the second aliquot of the saliva.

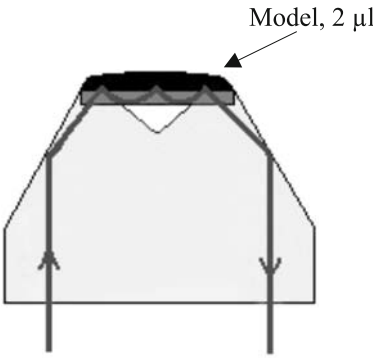

Fig. 1. ATR cell scheme.

Multivariate analysis using method of projections on latent structures was used for calibration model plotting for each saliva component. The spectra were adjusted to baseline and normalized. Spectral data processing was performed using Bruker Quant2 software (Bruker Optics).

RESULTS

To obtain stable and reproducible results of multivariate spectra analysis, 28 saliva samples were used for calibration within a range of concentrations attributable to normal physiological values of biochemical indices (Table 1).

We used various approaches for plotting of calibration models for total protein, amylase, glucose, cortisol, urea, sIgA concentrations. First, we used cross-validation procedure, when one spectrum is extracted from the calibration sample, while others are used for

TABLE 1. Mean Square Errors of Detection of Biochemical Indices in Saliva Samples

Index	RMSECV	RMSEP	Concentration range (calibration)	Concentration range (test)
Total protein, mg/ml	0.0416	0.0645	0.17-0.96	0.3-0.81
Glucose, μmol/liter	0.0318	0.00869	0.01-0.28	0.02-0.08
Urea, mmol/liter	0.187	0.14	2.5-5.9	3.8-5.6
Amylase, U/ml	55	89	44-830	13-827
sIgA, mg/liter	23.8	8.46	26-200	40-139
Cortisol, mmol/liter	2.21	3.1	5.42-24.74	9.77-18.87
Phosphate, mmol/liter	0.511	0.379	2.5-7.9	2.9-6.5

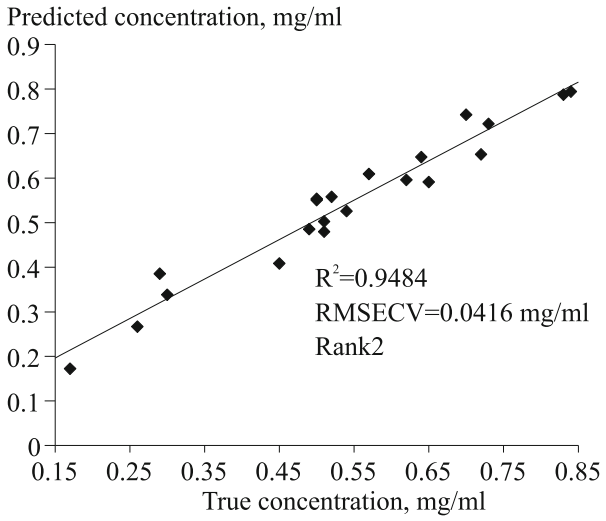

Fig. 2. Calibration model for calculation of saliva total protein level.

calculation of regression coefficient. Regression coefficients are then used for prediction of concentration in the extracted spectrum. Predicted value is compared to the value calculated used standard biochemical method. Mean square error of the predicted value is calculated as a root mean square error of cross-validation (RMSECV).

The second approach was applied to independent set of new samples, the test set. These spectra are not used for calibration. The concentration in these samples was predicted using calibrating model. Mean square error of predicted value is calculated as root mean square error of prediction (RMSEP; Table 1). Calibration models were plotted for eight saliva components.

Each saliva component possesses a set of specific absorption regions in IR range depending on the molecular composition and structure. The saliva is multi-component system, its spectrum is superpositional and reflects the impact of components present in the analyzed sample. Mathematic processing of spectral data enables indentification of changes of certain component basing on changes of saliva spectrum and its derivates in a specific range of wave parameters.

Calibration model plotted for calculation of total protein value in the saliva is shown on Figure 2. The model possesses good predictive properties, which is supported by high value of correlation coefficient R^2 (94.8%) and low RMSECV value (0.0416 mg/ml). Total protein content in saliva samples from the test set calculated using the plotted model is indicative of a high predictive strength of the model (Table 2), RMSEP value was 0.0645 mg/ml.

Calibration model, plotted for calculation of the urea concentration, also demonstrates high predictive ability (R^2=95.6%, RMSECV=0.187 mmol/liter), and urea concentrations, calculated by applying this model, are close to the values obtained using enzymatic colorimetric test, root mean square error of prediction (RMSEP) was 0.14 mmol/liter.

Spectral range with wave numbers between 1119 and 958 cm^{-1} was used for plotting the calibration model for calculation of glucose concentration in saliva samples. The indicated region contains pronounced absorption bands typical of valency oscillations of C-O and C-O-C bonds and variation of glucose concentration in the samples produce changes of spectral profile within this range. Characteristics of the plotted model: R^2=84.6%, RMSECV=0.0318 mmol/liter, RMSEP=0.00869 mmol/liter.

Normal concentration of inorganic phosphorus in the saliva is about 4.8 mmol/liter, it can also be calculated on the basis of spectral analysis. Calibration R^2 was 87.2%, RMSECV=0.511 mmol/liter, in test set RMSEP=0.379 mmol/liter.

Data on detection of sIgA and cortisol concentrations in test set of saliva samples are presented in Table 2. Predicted concentrations are close to the initial values, RMSEP is 8.46 mg/liter for sIgA and 3.1 mmol/liter for cortisol. Calibration models possess the following properties: R^2=78.2%, RMSECV=23.8 mg/liter for sIgA and R^2=79.4%, RMSECV=2.21 mmol/liter for cortisol.

These results indicate that precise detection of total protein level and concentration of such substances as glucose, urea, sIgA, cortisol, inorganic phosphorus, is possible. High precision is achieved by using a large

TABLE 2. Measurement of Total Protein (mg/ml), sIgA (mg/ml), and Cortisol (mmol/liter) in Control Samples of Saliva

No. of sample		Real	Predicted	Error, %
Total protein	1	0.6	0.5	17
	2	0.48	0.44	8
	3	0.81	0.76	6
	4	0.5	0.56	12
	5	0.3	0.35	17
sIgA	1	40	50	25
	2	105	116	10
	3	94	90	4
	4	75	67	11
	5	139	133	4
Cortisol	1	16.88	10.76	36
	2	18.87	18.49	2
	3	9.82	10.06	2
	4	9.77	12.78	30
	5	13.98	12.87	8

number of spectra for calibration (28 spectra in 10 repeats), which are scrupulously chosen according to physiologically normal values of various biochemical parameters of the saliva and which homogenously cover the whole calibration range.

The method based on ATR IR spectroscopy and mathematical processing of the spectral data using the method of projection on latent structures was developed as reagent-free analysis of saliva composition. Biochemical properties of the saliva can be detected without sample preparation (sample concentration, separation of detected components, removal of shielding agents) few minutes after sample obtaining. A sample of about 10 μl is necessary for detection of several components. Precision and reproducibility of detected parameters are comparable to routine clinical analysis and are limited only by reference analysis, which is used for plotting of the calibration model.

REFERENCES

1. A. B. Denisov. *Saliva and Salivary Glands* [in Russian], Moscow (2009).
2. G. Antonelli, E. Cappellin, R. Gatti, *et al., Clin. Biochem.,* **40,** No. 8, 545-550 (2007).
3. L. Benezzeddine-Boussaidi, G. Cazorla, and A. M. Melin, *Clin. Chem. Lab. Med.*, **47**, No. 1, 83-90 (2009).
4. C. E. Christersson, L. Lindh, and T. Arnebrant*, Eur. J. Oral. Sci.*, **108**, No. 5, 418-425 (2000).
5. G. Hosafci, O. Klein, G. Oremek, and W. Mantele, *Anal. Bioanal. Chem*., **387**, No. 5, 1815-1822 (2007).
6. D. Koh, V. Ng, L. H. Chua, *et al*., *Occup. Environ. Med*., **60**, No. 9, 696-698 (2003).
7. J. G. Lewis, *Clin. Biochem. Rev*., **27**, No. 3, 139-146 (2006).
8. M. Mulcahy, D. S. Evans, S. K. Hammond, *et al*., *Tob. Control,* **14** , No. 6, 384-388 (2005).
9. C. Rehbinder, J. Hau, *Can. J. Vet. Res*., **70**, No. 2, 151-154 (2006).
10. R. V. Santos, A. L. Almeida, E. C. Caperuto, *et al.*, *Comp. Biochem. Physiol. B Biochem. Mol. Biol*., **45**, No. 1, 114-117 (2006).
11. C. P. Schultz, M. K. Ahmed, C. Dawes, and H. H. Mantsch, *Anal. Biochem*., **240**, No. 1, 7-12 (1996).
12. D. Soo-Quee Koh and G. Choon-Huat Koh*, Occup*. *Envir. Med*., **64**, No. 3, 202-210 (2007).
13. N. P. Walsh, A. K. Blannin, A. M. Clark, *et al*., *J. Sports Sci*, **17**, No. 2, 129-134 (1999).
14. Y. P. Zhou, L. Xu, L. J. Tang, *et al*., *Anal. Sci.,* **23,** No. 7, 793-798 (2007).