Noninvasive Blood Glucose Assay by Near-Infrared Diffuse Reflectance Spectroscopy of the Human Inner Lip*

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Near-infrared (NIR) spectra of the human inner lip were obtained by using a special optimized accessory for diffuse reflectance measurements. The partial-least squares (PLS) multivariate calibration algorithm was applied for linear regression of the spectral data between 9000 and 5500 cm^{-1} (λ = 1.1-1.8 μm) against blood glucose concentrations determined by a standard clinical enzymatic method. Calibration experiments with a single person were carried out under varying conditions, as well as with a population of 133 different patients, with capillary and venous blood glucose concentration values provided. A genuine correlation between the blood glucose concentrations and the NIR-spectra can be proven. A time lag of about 10 min for the glucose concentration in the spectroscopically probed tissue volume vs. the capillary concentration can be estimated. Mean-square prediction errors obtained by cross-validation were in the range of 45 to 55 mg/dL. An analysis of different variance factors showed that the major contribution to the average prediction uncertainty was due to the reduced measurement reproducibility, i.e., variations in lip position and contact pressure. The results demonstrate the feasibility of using diffuse reflectance NIR-spectroscopy for the noninvasive measurement of blood glucose.

Index Headings: **Glucose; Near-infrared spectroscopy; Diffuse reflectance; Noninvasive monitoring; Multivariate calibration.**

INTRODUCTION

Blood glucose is an important parameter in medical diagnostics. More than 10% of the assays in an average clinical laboratory are concerned with glucose, making it the most frequently determined analyte in the hospital. A great number of measurements are carried out by patients suffering from diabetes mellitus, where periodic self-monitoring of their blood glucose levels is necessary in order to adjust calorie intake and/or administer insulin injections. In healthy subjects the glucose concentration is controlled in a closed-loop system provided by the insulin-producing β-cells of the pancreas, which also sense the current glucose level in the blood, so that the insulin secretion rate is increased within minutes after food intake, thus fixing the mean blood glucose concentration to a normal value of about 0.1 weight percent of blood or 100 mg/dL; whereas for diabetic patients with missing or insufficient insulin production, external control of the actual blood glucose level is required.

The development of a noninvasive, portable device for glucose self-monitoring is desirable for several reasons. For effective control, a type I diabetic patient often requires four and sometimes even more tests per day, with blood usually being taken from the fingertips. The removal of this daily constraint would considerably improve the patient's quality of life and, in this way, also strengthen his or her cooperation in achieving optimal metabolic control. The consequence of a noninvasive measuring device, allowing frequent measurements, is the possibility of a quasi-closed regulatory system with a nearly continuous watch on the patient's glucose level. The general health, medical care, and economic advantages lie in the long-term reduction in secondary physiological problems through the improved regulation of the blood glucose levels by more regular testing. Another concern is the reduction in the enormous costs of the disposable test strips which are required by the home monitoring devices currently on the market. Alternative methods are under investigation, and progress on invasive approaches for *in vivo* monitoring of glucose has been recently reviewed.[1]

Different approaches have been proposed to achieve the goal of an IR spectroscopic noninvasive blood glucose method which have also resulted in several patents.[2–5] However, only recent progress in spectrometry and chemometrics has led to acceptable results for the *in vitro* analysis of glucose in blood plasma.[6,7] Whereas mid-infrared spectroscopy using, in general, the attenuated total reflectance (ATR) technique can be applied successfully for the multicomponent analysis of several blood substrates including glucose, it lacks the penetration depth into tissue for a noninvasive measurement, necessary for monitoring blood glucose concentration transcutaneously. Alternatively, near-infrared spectroscopy can provide the penetration depth, at the cost of specificity due to fewer informative absorption signatures in the spectral ranges chosen for quantitative analysis. We have provided a comparison of the analytical performances for the use of Fourier transform spectroscopic data from either the long-wavelength NIR range or an optimized interval of the fingerprint MIR range, setting the bench marks for an IR-spectroscopic noninvasive assay for blood glucose.[6–8]

NIR experiments using radiation of shorter wavelengths around 1 μm can be carried out successfully in transmittance, even with a tissue layer thickness around 1 cm, since the absorptivity of the main tissue constituent (i.e., water) is reduced, although the optical scattering in the nonhomogeneous tissue will increase with shorter wavelength. The largest penetration depths are obtained

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within the so-called therapeutic window between 0.6 μm and 1.3 μm.[9] This spectral range was considered by Robinson *et al.*,[10] who made use of three different experimental setups including an FT spectrometer and grating monochromator with array detector, either with or without a fiber-optics accessory. As only oral glucose tolerance tests were performed during the preliminary investigation, the analytical relevance might be rather limited, because the pickup of spurious drift effects can influence the calibration results when a randomization of the measurements cannot be fulfilled. We experienced this problem when carrying out apparently sensitive ATR experiments with lip tissue of several diabetic probands using mid-infrared spectroscopy.

The study of the absorption spectrum of glucose initiated our *in vitro* transmittance and *in vivo* diffuse reflectance experiments. As the absorptivities of glucose around 1.6 μm (overtone bands of OH- and C-H stretching modes) are still of an acceptable magnitude, we decided to check the potential of this spectral range for quantitative multivariate calibration. To ascertain whether the penetration depth into tissue would be sufficient, we carried out Monte Carlo simulations, simulating the optical propagation of NIR photons in human tissue. By these means, different measurement conditions, either diffuse reflectance or transmittance, could be tested under varying tissue layer thicknesses. Due to energy limitations in the long-wavelength NIR, when transmittance experiments were performed, only a diffuse reflectance experiment turned out to be capable of reaching the penetration depth and the spectroscopic signal-to-noise ratios necessary for a noninvasive glucose assay.[7] To meet our spectroscopic requirements for a successful *in vivo* experiment, we constructed an optimized diffuse reflectance accessory.[7,11]

For a noninvasive measurement, the inner lip promised the best opportunities for realization, because the stratum corneum is missing and the mucous tissue found here is rather permeable for small molecules such as glucose; moreover, the lip is a homogenous tissue rich in capillary blood vessels and well thermostatted. In view of the pitfalls posed by spurious correlations to this extremely ill-conditioned measurement problem, different single-person calibrations as well as an experiment including 133 mainly diabetic patients were performed to evaluate the near-infrared spectrometric noninvasive assay, the results of which will be reported.

EXPERIMENTAL

Diffuse reflectance near-infrared (NIR) spectra of the human lower inner lip were obtained by having probands place their lips against the plane surface of a hemispherical immersion lens installed in a novel optical accessory and thermostatted at 37°C. The focus diameter of the illuminated sample area was about 2 mm. More details about the accessory have been published elsewhere.[7,11] The experiments were performed with the use of a Fourier transform IR spectrometer (Model IFS-66 from Bruker) equipped for NIR measurements (tungsten lamp, CaF_2 beamsplitter). The liquid nitrogen-cooled InSb detector with an element-diameter size of 4 mm was purchased from Infrared Associates (Suffolk, U.K.). A total of 1200 interferograms providing a spectral resolution of 32 cm^{-1} were averaged, and the resulting measurement time was about 1 min.

For the reference measurements, single-beam spectra were recorded with Spectralon reflectance standards from Labsphere (North Sutton, NH, U.S.A) pressed against the immersion lens of the accessory following careful cleaning. This material provides nearly ideal diffuse, Lambertian characteristics suitable for our reflectance application in the NIR spectral range. In the interval of 0.4 to 1.6 μm, the rather constant reflectivity of the white standard (SRM-99L-100C) is better than 0.98, and for longer wavelengths it decreases slightly ($R_{\lambda=1.8\mu m} = 0.976$, $R_{\lambda=2.0\ \mu m} = 0.957$, and $R_{\lambda=2.2\ \mu m} = 0.944$).[12] For the lip reflectance measurements, gray Spectralon materials possessing reflectivity values between 5% and 15% were used, so that a greater radiant power could be considered without saturating the detector signal or the ancillary amplification and digitization electronics. However, slight variations in reflectance were noticed when the diffuse reflectance standard material was pressed against the accessory lens, due to slight deviations in surface contact and changes in surface roughness. Much better reproducibility was achieved by using the Spectralon diffuse white standard drilled out cylindrically, so that a cavity resulted, providing a blurred image at the sample focus of the rotational ellipsoid used for collection of the diffusely reflected radiation. The radiant power reflected onto the detector was thereby reduced to a constant fractional rate required for adjusting the interferogram maximum. Best reproducibility for the reference measurements was obtained by this procedure.

Capillary blood samples of 20 μL for the glucose determination in the laboratory were taken by puncture of the fingertip with capillary pipettes from Brand (Wertheim, Germany). For the experiments with different patients from the clinical department of the Diabetes Research Institute, venous blood was also collected with the use of Monovettes from Sarstedt (Nürnbrecht, Germany). The hemolyzing reagent solution for the blood samples contained digitonin and maleimide. The blood glucose concentration was determined by a standard clinical enzymatic method (test combination Glucoquant Glucose from Boehringer Mannheim, Germany) with the use of hexokinase/G6P-DH,[13] which was available on an ACP 5040 analyzer from Eppendorf (Hamburg, Germany). Plasma samples from the venous blood charges were measured by a Boehringer Mannheim/Hitachi 704 analyzer by means of standard enzymatic methodology, providing concentration data for total cholesterol and triglycerides, which are of interest for judging the variation in composition of the sample population studied.

The partial least-squares (PLS) multivariate calibration algorithm was applied for a linear regression of the spectral data between 9000 and 5500 cm^{-1} (λ = 1.1–1.8 μm) against the probands' blood glucose concentration values. Mean-square prediction errors [PRESS$^{1/2}$ = $(\Sigma(C_{ref,i}-C_{pred,i})^2/M)^{1/2}$ with M samples; PRESS = predicted error sum of squares] were estimated by cross-validation using the "leave one out" strategy. Programs for the input of spectral data, PLS calibration, and cross-validation were written in MATLAB (The Mathworks, South Natick, MA, U.S.A). Use was also made of the

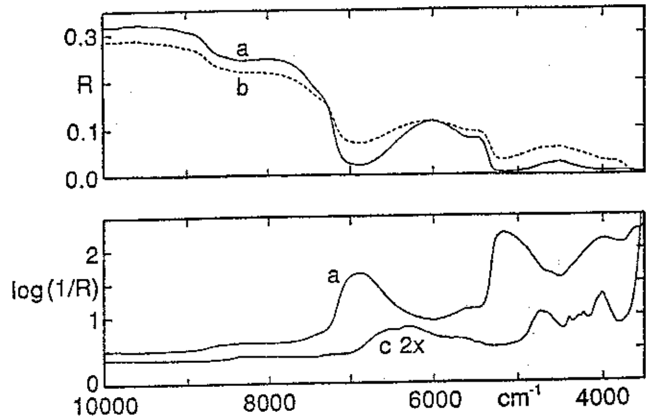

FIG. 1. Diffuse reflectance, resp. $\log(1/R)$ spectra for mucous lip tissue (trace a), for tongue tip tissue (trace b), and crystalline anhydrous glucose (trace c, enlarged for clarity).

signal processing toolbox. Further details to the calibration strategy including detection of outliers are described in Refs. 14 and 15.

RESULTS AND DISCUSSION

The diffuse reflectance accessory we used was particularly suited for measurement of bulky specimens. The high-throughput device was essential for the experiments planned. The refractive-index-matching immersion lens also promised reproducible sampling conditions, and the measurement time of 1 min was a compromise which could be tolerated by the patients. In the upper part of Fig. 1, two different reflectance spectra are shown; trace a gives a spectrum of the mucous tissue of the inner lip, whereas with trace b the spectrum of a tongue-tip is presented. Trace b is also an example of tissue not satisfactorily in contact with the immersion lens, so that Fresnel reflection contributes to the spectrum. The lower part of this figure gives the corresponding $\log(1/R)$ spectrum of the same lip tissue in comparison to a diffuse reflectance spectrum of crystalline glucose measured by the same device.

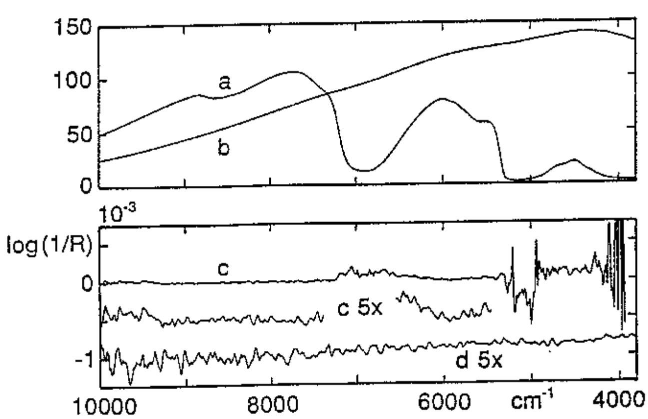

FIG. 2. Single-beam spectra of lip in arbitrary units (trace a) and a Spectralon gray standard with a reflectivity of about 0.13 (trace b) measured by the diffuse reflectance accessory (upper part); $\log(1/R)$ noise level estimated from two consecutive lip measurements (trace c) and single-beam measurements with the gray standard from above (trace d) (see lower part; the enlarged spectra are offset).

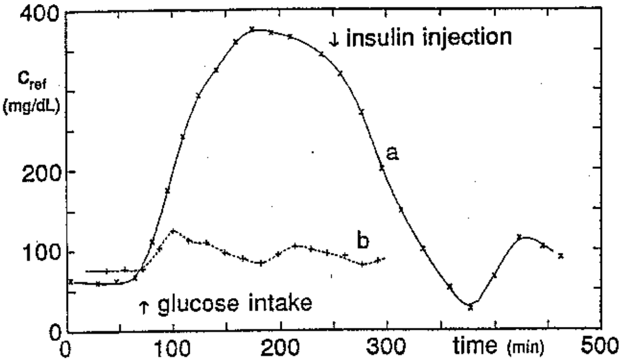

FIG. 3. Glucose time dependence for an oral glucose tolerance test (for details, see text) of a diabetic (solid curve) and a healthy test person (dashed curve); the same methodology was used for a calibration experiment with a single diabetic subject.

The energy limitations in the reflectance experiment for lip tissue are illustrated in the upper part of Fig. 2; trace a shows the single-beam spectrum of the FT spectrometer with lip tissue contacting the diffuse reflectance accessory, whereas for trace b a gray diffuse reflectance standard with a reflectivity of about 13% was used. In the lower part of Fig. 2, the corresponding $\log(1/R)$ noise levels for the use of two consecutive single-beam measurements either with lip tissue or the gray standard considered in trace b are displayed in traces c and d, respectively. The $\log(1/R)$ peak-to-peak noise level around 6000 cm^{-1} is smaller than $3 \cdot 10^{-5}$ absorbance units (AU). The glucose absorbance maximum at 6350 cm^{-1} measured for an aqueous solution (100 mg/dL) with a cell of 1 mm pathlength is about 10^{-4} AU, which underlines the ambitious goal of a noninvasive glucose assay.

First, experiments were performed with the use of oral glucose tolerance tests with supply of a sugar syrup (400 mL Dextro O.G.-T., Boehringer Mannheim, Mannheim, Germany), which is a mixture of mono- and oligosaccharides equivalent to 100 g anhydrous glucose after enzymatic cleavage. For the standardized test, three to six blood glucose determinations are usually carried out within the test duration of about three hours; the patient will start with an empty stomach. Figure 3 shows the response for a nondiabetic person (dashed curve), whereas the solid curve demonstrates the time dependence of the blood glucose concentration of a diabetic type I patient under these conditions, with the crosses representing the moments of blood sampling. The arrow near the glucose maximum indicates the time of an injection of regular insulin, leading to a diminished blood glucose concentration. For our experiments blood samples were taken from the test-person at time intervals of about 20 min. The concentration values obtained from these samples were interpolated with the use of a cubic spline function[16] providing the reference glucose values for three lip spectra recorded between two blood samplings made by puncture of the fingertip.

Multivariate calibration within a one-person experiment was carried out for a total of 133 spectral measurements taken during a two-day period with the blood glucose being monitored over 16 h. The blood glucose profiles were similar to Fig. 3; however, the insulin dosage was planned not to produce hypoglycemia, so that for the beginning and end of the test a normal glucose con-

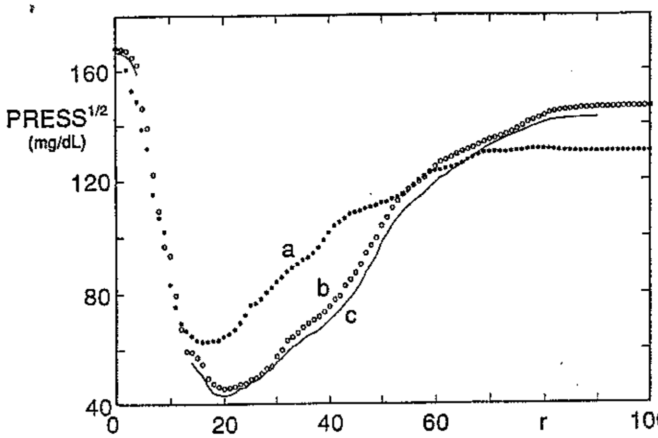

FIG. 4. PRESS^{½} statistics of the population mean-square prediction error for capillary blood glucose vs. number of PLS factors chosen with calibration data collected during a one-person experiment using oral glucose tolerance testing; results are from calibrations with 132 spectra of spectral range from 8994 to 5477 cm^{-1} with a wavenumber spacing of 30.8 cm^{-1} using log(1/R) (trace a) and logarithmic single-beam spectra (trace b); results of trace c (solid curve) are given for low-pass time-filtered glucose concentration values (time constant T = 10 min).

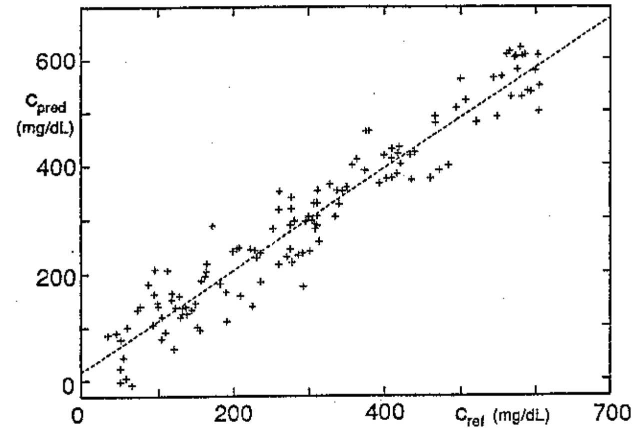

FIG. 5. Cross-validated predictions of glucose concentrations using the optimum PLS model of the noninvasive one-person experiment with oral glucose tolerance testing vs. interpolated glucose reference concentration values. Also given is the result of an *a posteriori* linear least-squares fit.

centration value of about 100 mg/dL resulted. In an effort to reduce the possible effect of spurious correlations, the sugar potion was split into two portions, so that intermediate plateaus, lasting for about one hour, appeared during the first day, with the result of a maximum glucose level of 420 mg/dL. For the second day, the total content of two syrup bottles was ingested, with the consequence that a maximum of 600 mg/dL was reached. The resulting reference concentration values are almost equally distributed between 30 mg/dL and 600 mg/dL, with an average value of $\bar{c}_{pop}$ = 301 mg/dL and a standard deviation of $\hat{\sigma}_{pop}$ = 167 mg/dL.

At first, calibration modeling was performed with the use of log(1/R) signals in an optimized spectral interval between 9000 and 5475 cm^{-1} with a wavenumber spacing of 30.8 cm^{-1}, giving N = 115 spectral data points. We noticed that spectral data pretreatment such as baseline correction, smoothing, or taking spectral derivatives did not improve calibration results. The rank-dependent average prediction errors (PRESS^{½}) are given in Fig. 4. It should be noted that the rank of the calibration matrices used here is equivalent to the number of PLS factors chosen for modeling. As a gray standard was considered (see also Experimental section) for the reference measurement, much prediction variance could be attributed to this spectroscopic step when the log(1/R) signals for calibration were taken into account.

This point becomes even more evident when we look at the results for multivariate calibrations presented in the same figure with the logarithm of the single-beam lip spectra being considered; these can be used, because the single-beam characteristic background is rather featureless and stable, although we experienced some variations in signal amplitude during the course of this experiment. Another advantage of the single-beam spectra, as compared to the log(1/R) spectra, is that the noise level will be reduced by a slightly wavenumber-dependent factor of about $\sqrt{2}$ (see also Fig. 2). A minimum of the average prediction error occurs at rank r_{opt} = 20 with PRESS^{½} = 45.6 mg/dL. In Fig. 5 a scatter plot of the predicted (cross-validated) concentrations vs. reference values is provided for the optimum model with respect to logarithmic single-beam data. The precision, especially for the lower concentration range, is rather poor; however, at high levels this limitation could be tolerated for a self-monitoring device for blood glucose. The *a posteriori* least-squares fit of the independent predictions vs. reference values gave c_{pred} = 17.0 + 0.945 c_{ref} with a coefficient of determination of R^2 = 0.92.

The tissue volume spectroscopically probed contains most of its glucose in the interstitial fluid of the intercellular space. Since the time dependence of the glucose concentration is known for this type of experiment (see Fig. 3), the time delay between the glucose concentrations in blood and probed mucous tissue, as expected for transport processes (e.g., by diffusion), can be simulated. A characterization of the glucose level in subcutaneous human tissue by microdialysis had been published recently.[17] For our delay simulation, the reference glucose signal, as approximated by the spline function mentioned above, was equidistantly sampled at intervals of one minute. A digital low-pass filtering was carried out by application of an impulse-invariant designed Butterworth filter of first order $y(n) = (1 - e^{-(1/T)})\, x(n) + e^{-(1/T)}\, y(n-1)$ with a time constant of T. As shown in Fig. 4, the filtering of the time-dependent reference concentration improves the prediction results, as the PRESS^{½} value decreases to 43.0 mg/dL for a time constant of T = 10 min. Extension to T = 20 min will cause a marginal deterioration in the result again (PRESS^{½} = 43.7 mg/dL at a calibration model rank r_{opt} = 19). The plausibility of our results is in agreement with the findings of Jansson *et al.*,[17] who found similar glucose levels under steady-state conditions, whereas for rapid changes a delay of 8 min for the glucose concentration in the subcutaneous interstitial space compared to that in venous blood could be experimentally determined.

For support of the results obtained by using oral glucose tolerance tests, a randomized one-person experiment with the same type I diabetic patient was carried out. During two weeks, three different, but rather constant, glucose levels (low, medium and high) were daily