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Near-Infrared Reflectance Spectroscopy for Noninvasive Monitoring of Metabolites

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An important class of substances in clinical chemistry are metabolites in body fluids, which are accessible by near-infrared spectroscopy without sample treatment using reagentless, fast and readily automated *in vitro* assays. Furthermore, noninvasive sensing systems are under development for the determination of blood glucose, especially for diabetic patients or for monitoring in intensive care and surgery. Near-infrared diffuse reflectance spectrometry of skin was employed allowing a certain tissue volume to be integrally probed. For calibration, the partial least-squares (PLS) algorithm was used either based on wide spectral intervals or using special spectral variable selection. Capillary blood glucose reference concentrations were obtained by finger pricking and an automated laboratory method (hexokinase/G6P-DH). Clear evidence is provided for the physical effect, as manifested by the spectral glucose absorptivities, underlying the individual single-person calibration models, which still require improvements in the methodology in the normo- and hypoglycemic concentration range. In extending the potential of noninvasive blood assays by infrared spectroscopy, a novel technique is presented for probing the intravascular fluid space by using fast spectral near-infrared measurements of skin tissue. The pulsatile blood spectrum can be derived from reflectance spectra of oral mucosa by Fourier analysis (near-infrared plethysmography). Future applications and prospects for noninvasive blood assays are discussed.

Key words: Near-infrared spectroscopy; Noninvasive glucose assay; Multivariate calibration; Self-monitoring of blood glucose; Pulsatile spectroscopy.

Abbreviations: ATR attenuated total reflection; DR diffuse reflectance; FTIR Fourier transform infrared; NIR near-infrared spectral range; OGTT oral glucose tolerance test; PLS partial least-squares; RMSEP root mean-squared error of prediction; SMBG self-monitoring of blood glucose; VIS visible spectral range.

Introduction

Spectroscopic methods are nowadays frequently employed for applications in clinical chemistry. An important category of substances is metabolites in body fluids, including glucose, which are accessible, for example, by a near-infrared spectroscopic measurement without sample treatment using reagentless, fast and readily automated *in vitro* multicomponent assays (1). Furthermore, research activities concentrate on noninvasive devices for self-monitoring of blood glucose (SMBG) or for monitoring in intensive care and surgery. *In vivo* glucose sensing and regulation is also necessary for patients with disorders of their carbohydrate metabolism, particularly diabetes mellitus. Self-monitoring is part of the daily routine for such patients, for which the measurement of capillary blood glucose still remains the standard method using test strip implemented enzymatic assays with photometric or electrochemical detection. Continuous measurements can be envisaged with implantable biosensors or ex-vivo devices coupled to microdialysis probes (2, 3). Infrared detection was also proposed for microdialysate analysis, and miniaturized devices will become available employing mid-infrared optical fibers (4, 5).

In general, noninvasive spectroscopic methodology exploits specific optical characteristics of the analyte, i.e. wavelength dependent absorptivities or refractive indices, mainly in the near-infrared (NIR) or, as for the optically active glucose, the rotation of linearly polarized radiation in the visible range (6–13). The visible and the short-wave NIR range exhibit absorption bands due to electronic transitions, whereas in the NIR with wavelengths from 780 nm to 2500 nm, molecules absorb significant radiation for excitation of overtone and combination vibrations. The photoacoustic measurement technique has also been employed and promising results were recently published by MacKenzie and coworkers (14). Other techniques currently under investigation are fluorescence and Raman spectroscopy (15–17). For recent publications reviewing this broad field of noninvasive approaches, see (18–21).

Near-infrared diffuse reflectance spectrometry of skin is the technique favored by us, which allows a certain tissue volume in the epidermis and dermis to be integrally probed. Although mid-infrared spectroscopy is very favorable for the analysis of blood samples, e.g. (22, 23), a measurement using the attenuated total reflection (ATR) technique lacks the penetration depth into tissue necessary for a transcutaneous measurement (24). On the other hand, an assay for whole blood was described using transmission spectroscopy around the 10 µm wavelength (in wave numbers 1000 cm^{-1}) (25). A noninvasive technique based on mid-infrared emission spectroscopy is under development by Optiscan (26).

Owing to the complexity of the integrally probed tissue, with the presence of many interfering compounds,

only multivariate measurement strategies using several wavelengths can be used for a noninvasive *in vivo* assay of glucose. Further complications arise from the heterogeneous distribution of glucose in the intravascular, interstitial and intracellular space and differences in their dynamics, contrary to the established reference methodology which employs capillary blood. Our recent analyses of results with diffuse reflectance spectroscopy of the inner lip of a single diabetic person based on different strategies are presented, which give confidence in future developments of reliable and robust glucose sensing devices. Furthermore, the noninvasive probing of the intravascular space can be tackled by pulsatile multivariate spectroscopy. First promising results are presented. However, the implementation of such a technique, in particular for metabolite monitoring, requires a spectral signal-to-noise improvement by an order of magnitude or more compared to integral tissue probing, currently considered for transcutaneous glucose monitoring.

Subjects and Methods

Experimental

NIR-spectroscopy is a widely applied technique for obtaining quantitative results on a great variety of substances, in particular, for applications in agriculture and food science. With the advent of multivariate calibrations, the technique has been tremendously successful within the field of routine analysis and process control. A particular challenge observed in medical applications is the reduced concentration range of compounds to be monitored against an extremely large and varying background.

Using spectrometry within the so-called therapeutic window with wavelengths between 0.6 µm and 1.3 µm, a transmission measurement, for example, of a finger is feasible. Apart from such NIR-transmission measurements, radiation scattering experiments using diffuse reflectance (DR) of skin are also possible in the long wavelength NIR spectral range. Due to increased absorption coefficients from water, the main constituent of tissue, the penetration depth for such radiation is reduced compared to that in the therapeutic window. A critical aspect is the penetration depth for photons with wavelengths around 1.6 µm, where glucose absorptivities are large enough to be considered for *in vivo* measurements. This fact has also been supported by Monte Carlo simulations modeling the photon transport in tissue (27, 28).

Mucosal lip tissue was chosen, because it is rich in capillary blood vessels and allows a splendid optical contact to the DR-accessory constructed by us for recording high quality *in vivo* spectra. Further studies were carried out using different body skin measurements in the visible and short-wave NIR spectral ranges, which supported the fact, that the largest blood volume can be sensed in such mucosa compared with other tissues. This is demonstrated with the diffuse reflectance skin spectra of a single person shown in Figure 1. The spectra were measured with a Multiscan-OS10 spectrometer (NIOS GmbH, Germany) equipped with a fiberoptic probe. The oxyhemoglobin doublet band around 560 nm dominates the skin spectra, and the band height can be taken as a relative measure for the blood volume within the tissue probed.

NIR-spectra of the inner lip were recorded using a Fourier Transform spectrometer (model IFS-66, Bruker Analytische Meßtechnik, Karlsruhe, Germany) equipped with tungsten lamp and CaF_2 beam splitter. The InSb detector (Infrared Associates, Suffolk, UK) was placed in the DR-accessory (27). The design is based on an on-axis ellipsoidal mirror collecting the diffusely reflected radiation from the lip tissue in a much more efficient manner than is possible using commercially available accessories. Emphasis had been placed on the measurement of skin tissue with a minimum of inconvenience for patients while achieving an extremely high signal/noise ratios and spectrum reproducibility for the tissue studied. As temperature is a critical parameter when measuring biotic aqueous systems, provision for thermostating the contact material of the accessory at 37° C was taken into account. For calculating reflectance spectra ($R = I_R/I_0$) it was necessary to record reference spectra by means of standards of different reflectivity made by Spectralon (Labsphere, North Sutton, NH, USA).

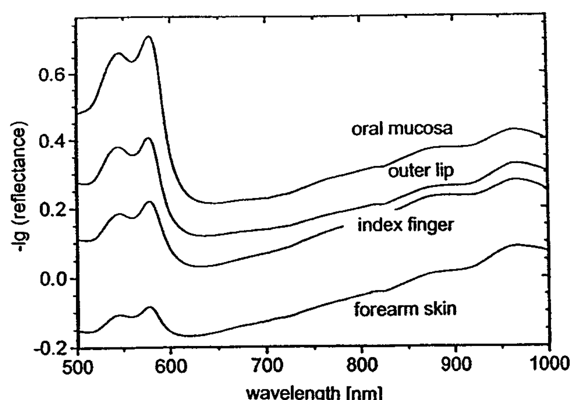

Fig. 1 Reflectance VIS/NIR spectra of different skin tissues recorded at normal skin temperature by using a bifurcated fiber optic probe (for clarity, the individual spectra are offset).

The lip spectra were recorded by placing the inner lip against the immersion lens of the DR-accessory. Measurement time was about 1 minute to accumulate 1200 single sided interferograms, equivalent to a spectral resolution of 32 cm^{-1} after Fourier transformation. The absorbance equivalent noise level achieved with application of a grey Spectralon standard of 10% reflectance is shown in Figure 2A, which can be estimated from the logarithmic transformation of the ratio of two subsequent measurements. The top part of this figure displays a logarithmized DR-lip spectrum as measured against a Spectralon standard of nearly 100% reflectance. Our optimized DR-accessory yields further benefits, as the maximum absorbance equivalent values of the measured lip tissue are below 2.0, so that the absorbance noise and photometric errors, which are generally exaggerated in spectral sections with absorbances greater this limit, are not a problem for the computation of robust calibration models (29).

Complementary NIR-studies were also carried out using a bifurcated, Y-shaped fiberoptic probe, with quartz fibers for illumination and collection arranged at random (single fiber diameter of 0.2 mm, total length 1.5 m, common end diameter of 4 mm; supplied by Bruker Optik, Karlsruhe, Germany). A comparison between the spectra obtained by the DR-accessory and the fiberoptic probe is presented in Figure 2B. Due to the numerical aperture of the fibers only back-scattered photons within a small limited solid angle can be collected. Those photons have proportionately more deep penetration depth into the skin tissue, which can be estimated using transmission equivalent water band absorbances. The logarithmized reflectance values unfortunately show rather high values be-

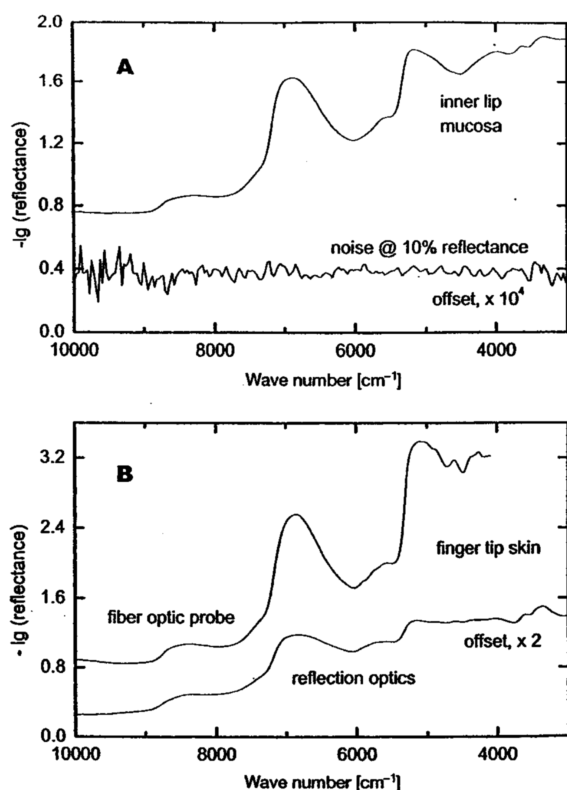

Fig. 2A Diffuse reflectance (DR) spectrum of inner lip tissue as measured versus white Spectralon, and -log R noise level estimated from two subsequent measurements using a grey Spectralon standard of 10% reflectance.
B Diffuse reflectance spectra of the finger tip skin as measured with a bifurcated fiber optic probe and the DR-accessory, based on an on-axis ellipsoidal mirror.

low the spectral region of 7000 cm^{-1}, apart from a narrow window with values below 2.0. A positive aspect in using such a fiberoptic probe is that the *stratum corneum*, e.g. of the finger tip skin, does not diminish the signal, which is not the case for the DR-accessory, as can be seen from the lower spectrum trace. For both probes the reflectance in the spectral interval between 10000 and 9000 cm^{-1} is comparable in value.

A further development of noninvasive assay technology is based on time-resolved near-infrared spectroscopy allowing the probing of the intravascular fluid space, since its arterial volume is modulated by the heart beat, which can be sensed by rapid spectrum acquisition. For the time-resolved measurements, the spectrometer software for gas chromatography/FTIR coupling was employed. A total of nine interferograms with the same resolution as given above were averaged within 0.5 s. The diffuse reflectance lip spectra were recorded for a measurement time of at least two minutes using the optimized DR-accessory. Subsequent background spectra were measured using Spectralon. The spectra were transfered to a personal computer for further processing using MATLAB (The Mathworks, South Natick, MA, USA).

Reference methodology and calibration package

Reference blood glucose concentration values were obtained by standard enzymatic methodology (Test Combination Glucoquant Glucose, Boehringer Mannheim, Germany) with the use of hexokinase/G6P-DH available on an ACP 5040 analyzer from Eppendorf (Hamburg, Germany). The capillary blood samples were taken with 20 µL capillary pipettes from Brand (Wertheim, Germany) after finger pricking. Prior to the spectral lip measurements, the patient was asked to rinse his mouth with mineral water.

As the timing of the reference blood samplings did not coincide with those of the spectrum recordings, and the time gap between each reference measurement was about 15 minutes, spline approximations between reference concentrations were calculated by fitting a cubic spline function. The signal processing toolbox of MATLAB was employed for time filtering of glucose concentration profiles obtained during oral glucose tolerance testing; see also (36, 37). The effect of time filtering by applying an impulse-invariant designed Butterworth filter of first order with, for example, a time constant of 10 minutes, was used to simulate glucose diffusion and other transport processes from the intravascular to the interstitial space (38). This is important, because the spectrometric assay results cannot be exactly correlated to the vascular capillary glucose concentration due to the integral tissue probing.

Multivariate calibration was carried out using a partial least-squares (PLS) PC-software package programmed in MATLAB. Instead of splitting the data into calibration and validation subsets, which is a reasonable strategy when a large number of standards is available or the complexity of the calibration problem is low besides an appropriate spectral quality, cross-validation is an alternative strategy to be favored with extremely sensitive applications, to make best use of the available data. The results are equivalent for large standard populations, which has been proved by us and other groups (30, 31). For optimum calibration model selection, such cross-validation with "leave-one-out" strategy was used with the following procedure. One standard is removed from the calibration set, regression is carried out, and the concentration is predicted for that single standard. These steps are repeated, until all standards are considered. Also groups of several standards at a time were tested for independent prediction, evaluating the robustness of the calibration models. The root mean-squared error of prediction (RMSEP = $(\Sigma\ (c_{ref,i} - c_{pred,i})^2/M)^{1/2}$ with M samples) is a suitable statistic for optimum calibration model selection (32, 33). Apart from full spectrum interval regression, also a special wavelength selection procedure was chosen, which renders improved and more robust statistical models for single person calibrations. The algorithm is based on a pairwise selection of extreme values, i.e. minimum and maximum, of the optimum regression vector obtained from previous calibrations based on equidistant spectral variables within the intervals previously optimized; for details see (34, 35).

Patients and calibration design

The experiments reported were in accord with the ethical standards for human experimentation. All single-person experiments were carried out with a type 1 diabetic male patient either using oral glucose tolerance tests (OGTT) during a two-day trial or, for further verification, testing of random glucose levels over a period of two weeks. Each OGTT was started in the morning, after which Dextro O.G.T. (Boehringer Mannheim, Germany), equivalent to 50 g of anhydrous glucose after enzymatic cleavage, was ingested raising the blood glucose level within about 1 1/2 hours. Some additional carbohydrate (1st day 50 g, 2nd day 150 g) was consumed so that blood glucose reached a maximum. Insulin was then administered later (1st day 10 IU, 2nd day 14 IU), resulted in the glucose concentration dropping to low values within about 1 1/2 hours. Total time duration for the OGTT-experiments was 15 hours, gather-

ing a total of 133 lip spectra. The reference concentrations within the two-day experiment were equally distributed between 30 mg/dl and 600 mg/dl (1.7–33.3 mmol/l) (population average c_{av} = 301 mg/dl (16.7 mmol/l), and standard deviation σ = 167 mg/dl (9.3 mmol/l).

The testing at different glucose levels was carried out over two weeks. Three different daily glucose plateaus of low, medium and high concentration were planned, the order of which was selected at random. The levels were checked by an SMBG device (Reflolux II, Boehringer, Mannheim, Germany). Each experiment lasted about 40 minutes and consisted of three blood samplings with three repeat lip spectra recorded in between two subsequent tests, with another spectrum taken before and after the blood collection procedure, giving 8 spectra in total. The glucose concentrations as determined by the hexokinase/G6P-DH method in duplicate were used for interpolation by a cubic spline function to yield the actual glucose values at the time a spectrum was recorded. The range of concentration obtained for this calibration experiment with 219 lip spectra was again from 30 to 600 mg/dl (c_{av} = 269 mg/dl, σ = 162 mg/dl).

Results and Discussion

Noninvasive glucose assay based on single-person calibrations

For multivariate calibration, the selection of appropriate spectral intervals is essential, although the algorithms applied are considered to be full-spectrum calibration methods. Reasons for omitting a particular section of the spectrum are that its noise level varies significantly, or spectral variance from a major constituent exists which cannot be modelled satisfactorily. For these *in vivo* calibrations the spectral interval between 9000 and 5450 cm^{-1} was chosen. The region below 5450 cm^{-1} was omitted due to small radiation penetration depth and the increased spectral noise owing to the low reflectance. The upper wave number limit of 9000 cm^{-1} was chosen, because the slight gain of glucose information obtainable above this limit is offset by random spectral effects.

Calibration models were calculated by using logarithmized single beam lip spectra only. This approach was very successful, since no noise-containing background spectrum is required as for absorbance equivalent data; see also (1, 39). Preprocessing of the spectral data such as smoothing, taking spectral derivatives or baseline corrections did not improve the prediction performance as compared to raw spectral data (40).

In Figure 3A the PLS rank dependence of the prediction error of different calibration models for the one-person OGGT experiments is shown based on 115 equidistant spectral variables within the spectral interval discussed above, and a delayed glucose concentration profile (time constant 10 minutes; removal of one outlier). The root-mean-squared prediction errors are presented for one or packages of five standards considered for cross-validation, respectively. A minimum is reached for a PLS rank of 20 (leave-one-out RMSEP: 43.0 mg/dl; leave-five-out RMSEP: 46.9 mg/dl). By applying our spectral variable reduction procedure, 26

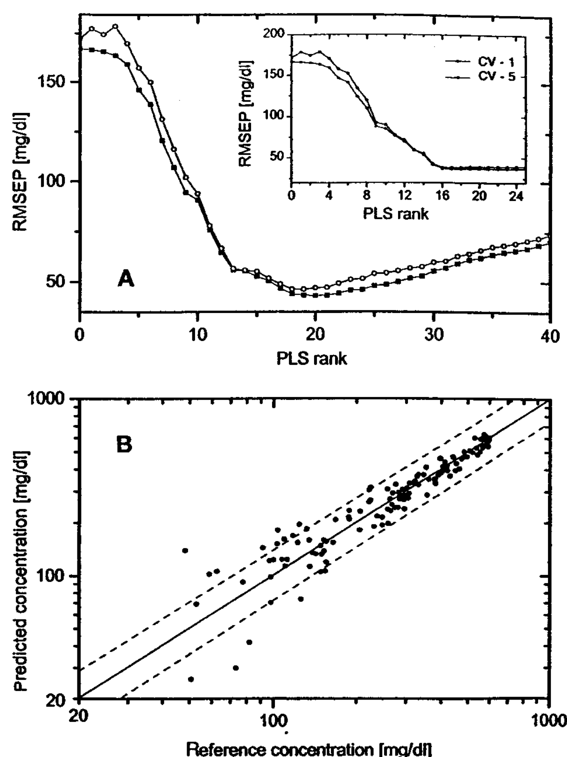

Fig. 3 Calibration results for blood glucose using 132 diffuse reflectance lip spectra obtained from a single-person experiment with non-standard oral glucose tolerance testing and delayed glucose concentration profiles (see text):
A root mean squared error of prediction (RMSEP) with full spectrum evaluation versus PLS model rank; the leave-one-out cross-validation (CV-1) results are given by squares, results from leave-five-out (CV-5) are illustrated by open circles); the subplot provides the results from different PLS models based on 26 selected spectral variables.
B predicted concentrations versus reference values based on 26 variables with an optimum PLS rank of 16 (the confidence interval for a 30% relative standard deviation is marked by dashed lines). To convert results (mg/dl) to mmol/l divide by 18.

specific wavelengths were selected (see also (7, 8)), for which the optimum PLS model is found with 16 PLS factors (RMSEP = 36.4 mg/dl; see inset in Figure 2A). Cross-validation with packages of five standards leads to only a slight increase in the RMSEP-value by 1.8 mg/dl, illustrating the improved robustness of such a model compared to the broad interval calibration with 115 spectral variables. In Figure 3B a scatter plot of the NIR-predicted glucose concentrations versus the delayed blood glucose reference concentrations is shown using double logarithmic presentation, illustrating the current deficits of the NIR methodology. Only a small bias is observed (c_{pred} = 11.5 + 0.962 c_{ref}) and a high coefficient of determination (R^2 = 0.952) is found. However, as exemplified by the relative confidence interval of ± 30%, the scatter in the normo- and hypoglycemic concentration range is significantly above this band. In this context, it must be remembered that the clinically

relevant glucose concentration range is from 30 mg/dl to about 350 mg/dl.

A similar calibration was carried out for the data recorded during the two-week tests with the exception that no delayed glucose concentration profiles could be calculated due to the short duration of daily testing. For this calibration experiment 32 spectral variables led to a RMSEP-value of 46.8 mg/dl (for a calibration model with 26 spectral variables a slightly raised value of 48.5 mg/dl was achieved). This must be compared against the 115 spectral variable calibration model with 22 PLS factors which reached a RMSEP-value of 51.9 mg/dl for leave-one-out and 55.7 mg/dl for leave-five-out cross-validation, respectively.

The plot, as shown in Figure 3B, does not display information with respect to systematic deviations, although these seem not to exist when looking at the prediction scatter. However, the diagram of time-dependent pairs of NIR-predicted and reference glucose concentration values (see Figure 4A) displays that differences are obvious for the start of the OGTT test of the first day, where a diminished tissue glucose concentration can be regarded as plausible. A similar discrepancy is evident for the plateau maximum of the second day, which gives the impression that the integral tissue glucose is kept at a constant level, although the blood glucose values are already falling. In Figure 4B a similar plot is provided for the glucose pairs from the 4th day up to the 7th day, where the different daily tests are separated by a time gap of 60 minutes to allow a compressed presentation. It clearly demonstrates the necessity for patient clamp experiments to improve the reference methodology, although certainly also the reproducibility of the skin tissue measurement and the spectral outlier identification are strategies to be considered in future research.

The spectral data of the two-week-testing were studied in detail providing more clues for improvement. In Figure 5A, trace (a) the mean -log R spectrum is presented together with the population standard devia-

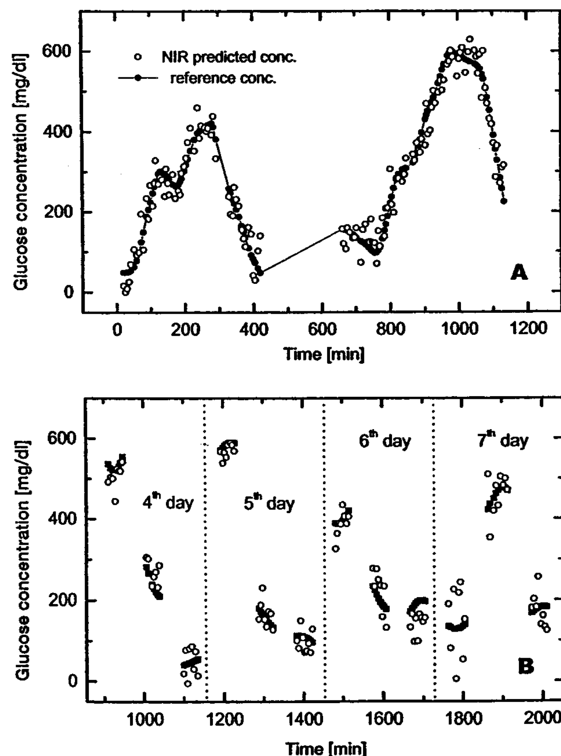

Fig. 4 Comparison of time-dependent capillary blood glucose concentration values (calibration reference data) and prediction results based on PLS calibration using diffuse reflectance lip spectra obtained in experiments with a single diabetic person:

A Results for the two-day non-standard oral glucose tolerance testing (see also Figure 3);

B Representative section of results obtained for four days within a two-week testing experiment: three sessions with different blood glucose levels randomly selected were chosen during the whole day (for compressed presentation the time gap between the different sessions is deliberately reduced).

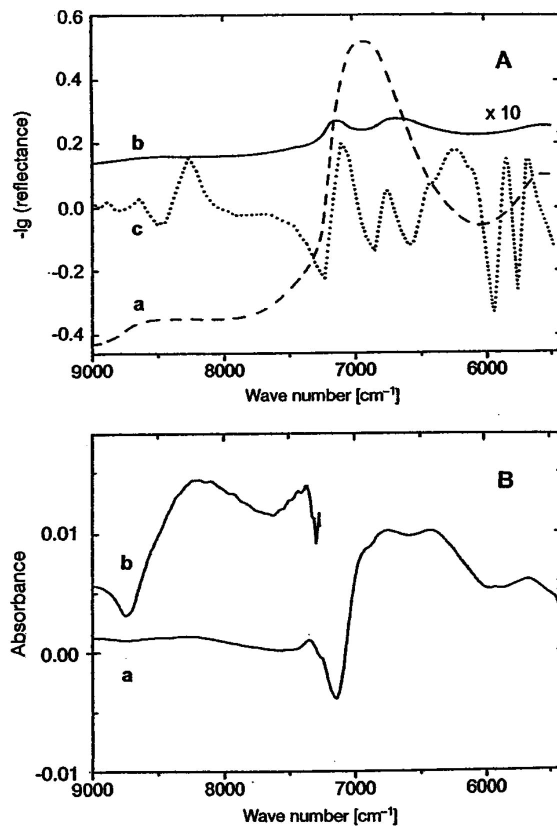

Fig. 5A Spectral calibration data for the one-person experiment with random testing over a two-week period: population mean spectrum measured against a grey reflectance standard (a), spectral population standard deviation (b) and regression vector (arbitrarily scaled) derived from 10 PLS factors (c);

B Glucose absorbance spectra within the spectral range considered for a noninvasive spectrometric assay: difference spectrum of a 5.0% glucose solution as measured in a 0.5 mm cell (a), and difference spectrum of a 2.5% glucose solution as measured in a 10 mm cell (b), both at a temperature of 30°C and with water absorbance compensation (the latter trace is offset for clarity).

tion, see trace (b), and a regression vector calculated from 10 PLS factors (c). Further factors needed to achieve optimum prediction will model this vector by adding higher frequency components. However, the intermediate evolving structure can be directly related to the absorptivity data of aqueous glucose, which is shown in Figure 5B. Similar extreme values for the PLS regression vector with the number of variables reduced were also derived, e.g., for the two-day OGTT-calibration experiment; see also (8).

The quality of the statistical calibration models based on oral glucose tolerance testing has been questioned recently (31). It is well known that pickup of spurious drift effects can significantly influence the results from multivariate statistical calibrations, especially with regard to continuous monitoring. An appropriate experimental design is necessary for such critical applications when the analytical signals are comparable to the prevailing noise level or signal drift. Our strategy in the past was able to avoid temporal chance correlations. When we compare the population standard deviation of the glucose concentration values in our two single-person calibration experiments with the RMSEP-values achieved with the optimized PLS calibration models, we obtain significant values of 4.6 and 3.5 when taking the ratio of both values for the two-day and the two-week experiments, respectively.

Arnold and coworkers (31) evaluated phantom tissue NIR measurements to those deliberately, without any glucose present, but assigning one of our OGTT-glucose profiles. When these authors employed a reasonable experimental design, e.g. cross-validation and a spectral resolution of 32 cm^{-1}, they achieved a RMSEP of 4.16 mmol/l (74.9 mg/dl) compared to the standard deviation of the reference glucose concentrations studied of 7.09 mmol/l (127.6 mg/dl) providing a quotient of 1.7 for the ratio, as described above. Simple F-testing as applied in these cases must be questioned, as it does not exclude the possibility of spurious correlations. Similar experiences as described by Arnold *et al.* were made by us when mid-infrared ATR lip spectra were used for PLS calibrations (24), supporting our point of view that such a technique is not appropriate to be used for a noninvasive glucose assay. From our experiences with sensitive statistical spectroscopic calibrations, it is advisable to test the robustness of the PLS models by choosing a different package size within the frame of cross-validation. In addition to a well designed calibration experiment, the regression vector shape should be studied, providing evidence for the physical effects (i.e. glucose absorptivity) underlying the noninvasive assay using the diffuse reflectance technique for the measurement of skin tissue.

Further contributions were recently presented by Danzer *et al.* for improving a noninvasive glucose assay using NIR-spectroscopy of skin (41, 42). However, their strategy was based on outlier removal to an extent that the calibration population was drastically diminished, which leads to problems for implementing this into routine assays.

Finally, the selectivity of the chosen approach will be discussed. For the *in vitro* studies (1, 18) satisfactory quantitative results based on a multi-person calibration within a hospital study were achieved by using long wavelength NIR data. By picking the shorter wavelength spectral range between 900 and 1300 nm, the band halfwidths are usually increased, so that a reduced selectivity for appropriate tissue transmittance measurements can be expected compared to experiments with wavelengths around 1600 nm. In this context, more work is required to identify possible interferences from, e.g., pharmaceutically active substances which may influence the spectrometric glucose measurement; for further discussion, see (43).

Near-infrared plethysmography

Integral tissue probing suffers many limitations as described in detail (18). As most clinical parameters are obtained by the analysis of blood, it is highly desirable to have access to similar information by noninvasive spectrometric means. However, since the blood volume represents only a small fraction of the total skin

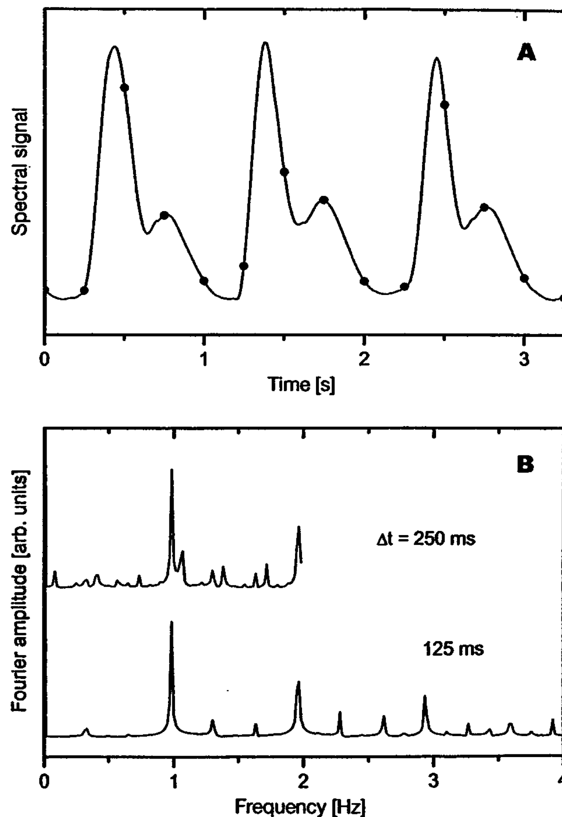

Fig. 6A Signal of the cardiac-modulated remission of the skin, observed, e.g., for hemoglobin absorption at 580 nm due to arterial blood volume changes (for later Fourier analysis the signal was digitized every 250 ms as illustrated by dots);
B Amplitude frequency spectrum obtained by a Fourier transformation of a time-extended trace as shown above (for the lower trace: due to halving of the sampling time, the maximum Nyquist-frequency is doubled, by which aliasing problems can be reduced).

tissue probed, the signal changes due to the pulsatile blood flow are minimal when compared to the total tissue water. Such a measurement principle, which was recently reviewed (44), has been applied in pulse oximetry for many years. A Fourier analysis of a pulse oximetry signal trace, e.g., using skin absorption in a diffuse reflection experiment below a wavelength of 600 nm (see Figure 1) and sampled equidistantly in time, provides us with the correct frequency to determine the fundamental amplitudes of the cardiac-modulated periodic blood volume variations (see Figure 6). For a repeated sampling time of 250 ms, some overtone frequency components are folded at the corresponding maximum Nyquist-frequency into the frequency interval available after Fourier transformation, giving rise to a so-called aliasing problem. When the sampling time interval is shortened, as also shown in Figure 6B, the fundamental frequency component here, but also in the trace above, can be clearly isolated from other overtone contributions. However, this technique has not yet been applied for metabolite measurements due to limitations in spectral signal-to-noise ratio, so far observed for *in vivo* near-infrared measurements.

Results of time-resolved measurements on human

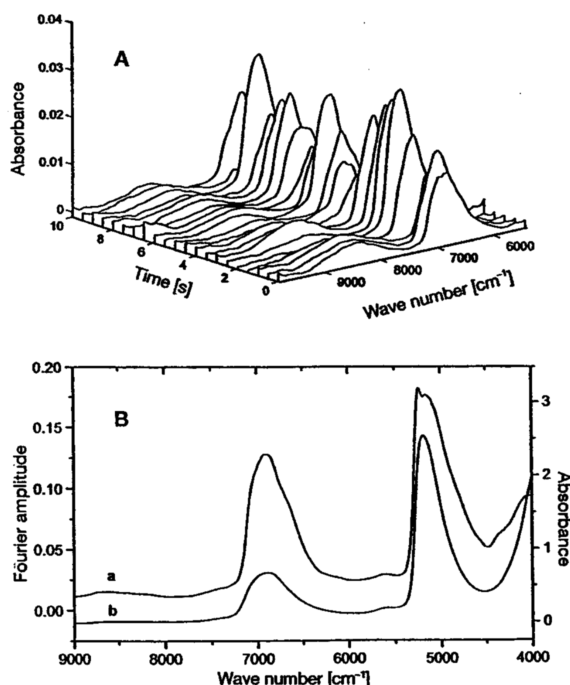

Fig. 7A Some time-resolved inner lip spectra of a single person shown as differences versus the first measured single beam lip spectrum after Savitzky-Golay smoothing;
B Fourier amplitudes (trace **a**) illustrate the relative pulsatile spectral components in the near-infrared diffuse reflectance spectra of human oral mucosa due to cardiac-modulated blood volume variations (for each spectral variable the Fourier amplitude coefficients were averaged around the heart beat frequency within a frequency interval of 0.06 Hz); for comparison the absorbance spectrum of water as measured in a 0.5 mm cell is given below (trace **b**, right ordinate scale).

oral mucosa are presented using diffuse reflectance spectroscopy. The first individual lip spectra obtained for a demonstration experiment with fast measurements are shown in Figure 7A. These are difference spectra smoothed for noise reduction by a Savitzky-Golay polynomial, as calculated versus the first measured spectrum of the data set, after application of a polynomial baseline fitted to predefined spectral intervals which are located in the spectrum minima. It clearly illustrates the intensity fluctuations caused by changes in the arterial blood compartment associated with the cardiac cycle. The logarithmized single beam lip spectra were later preprocessed by a Savitzky-Golay smoothing with a quadratic polynomial of 25 data points.

Each Fourier analysis of the time-dependent logarithmized single beam intensities, assigned to the individual spectral variables, provides us with the spectral Fourier amplitudes, in particular at the fundamental heart beat frequency. Considering a cut through a three dimensional diagram with spectral wave number variables, frequency components in Hz and pulsatile amplitudes as ordinates, along the heart beat frequency leads to the pulsatile spectrum. This has no static component, as originating from the aqueous unmodulated compartments of the skin tissue; for more details, see (8, 45). The pulsatile spectrum showing only component changes with pulse is a kind of difference spectrum (see Figure 7B), and it is similar to the water absorbance spectrum as recorded with a transmission cell of 0.5 mm pathlength. The water absorbance alterations due to the cardiac blood pressure changes are about 20 mA.U. for the water band at 6900 cm^{-1}. This is equivalent to a water layer of 15 μm thickness, which is about a factor of 50 smaller than obtained for the integrative measurements discussed above. In comparison to the human plasma study for which a cell of 1 mm pathlength was applied (1), there is even a factor of 70 between the corresponding water absorbance values. It is noteworthy, that the ratio of the maximum amplitudes of the water combination band at 5200 cm^{-1} and of the overtone band at 6900 cm^{-1} is much smaller for the pulsatile spectrum than for the water absorbance spectrum recorded in transmission. This can be explained by the significantly different penetration depths for the near-infrared radiation realized for those wavelengths.

Conclusions

Accurate knowledge of the blood glucose concentration is essential for the therapy of diabetic patients. For the last 20 years, small portable devices for self-monitoring of blood glucose (SMBG) have been commercially available, which require invasive blood sampling, preferably from the finger tip, using for example, a lancet. These devices represent significant progress within diabetes control, although the disadvantages of high cost for reagent strips and, in particular, of the patient anxiety and discomfort in relation to finger prick-

ing exist. Noninvasive NIR-spectroscopic techniques will allow frequent or even constant monitoring of blood glucose levels, which is certainly not possible with self-monitoring devices currently available.

Using a special spectral variable selection based on choices from the optimum PLS regression vector yields better results than using the PLS calibration models with full spectrum evaluation previously reported. An important aspect is the reduced number of spectral variables needed for robust calibration modeling. Evidence is provided for the physical effect, as manifested by the spectral glucose absorptivities, underlying the individual single-person calibration models. Their regression vector structure shows very similar features as calculated for a glucose calibration experiment based on random human plasma samples (8, 35). The problems in calibrating skin tissue spectra versus blood glucose concentration values need further discussion, exemplifying the need for more knowledge about the dynamics of glucose exchange from the vascular system into the interstitial and intracellular space. On the other hand, additional relevant information on the metabolic status and microcirculation can be gained by spectral measurements of skin in the visible and short-wavelength near-infrared spectral range (oximetry).

Novel techniques for the analysis of blood substrates were presented for probing the intravascular fluid space using fast spectral near-infrared measurements, such as used in pulse oximetry. A pulsatile component blood spectrum was derived from diffuse reflectance spectra of oral mucosa by Fourier analysis (near-infrared plethysmography).

Further development aimed at continuous monitoring devices for the determination of blood glucose is an ambitious goal in analytical technology. Miniaturization techniques have made important strides in recent years and micromachined instruments are available in the diabetes therapy field, e.g., insulin pumps. Possibilities are also arising in the design of small spectrometers profiting from very large scale integrated electronics. However, present spectroscopic technology and chemometrics still require further improvements. Technology transfer from the noninvasive blood and tissue oxygenation monitoring techniques might provide further scientific impetus for research into noninvasive glucose assays by NIR spectrometry.

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