

It Will Just Need a Drop of Blood

Reagent-free Laboratory Diagnostics in Medicine Based on Infrared Spectroscopy

If you consult a doctor or enter a hospital for a test of for some treatment, blood or urine samples will be taken in most cases that are analyzed in a laboratory. In a big hospital, analysis will be done in a specialized laboratory unit. In smaller hospitals or at your family's doctor, these samples are typically sent by express delivery to a laboratory service. The pressure to reduce health care costs has led to a real "sample tourism" with increasing traveling distances and time lapse between taking the sample and the availability of the blood or urine parameters. Consequently, this may lead to delays for therapies and in turn reduces overall medical efficiency.

It is for this reason that health care management more and more aims for a bedside point-of-care-testing where a quick analysis of blood or urine parameters is performed. While this would improve considerably patient supervision on intense care units (ICU), it is equally important in smaller hospitals, in long-term care units for multi-morbid patients or for elderly people. Although there are existing test kits, they are rarely used either because of their high costs or insufficient precision. On the other hand, there is also an enormous potential to reduce costs in large clinical laboratories, simply by the fact that the daily number of blood and urine samples in a large hospital may easily reach several thousands. The costs for reagents used in automated analysis, the daily calibrations required, and in particular costs for labour are a main factor for cost efficiency in health care management.

Most procedures for the analysis of full blood samples or blood plasma samples or rely on a spectroscopic measurement in the visible or UV

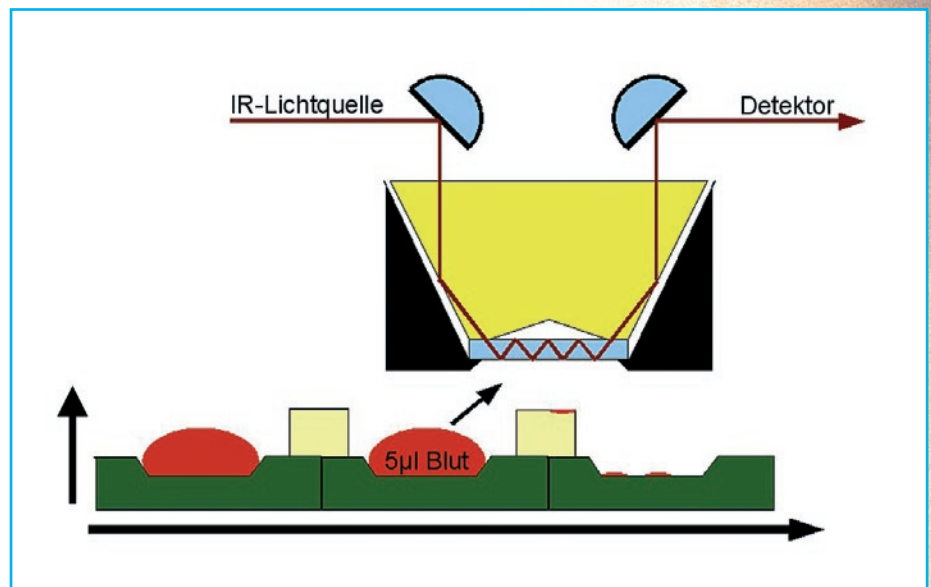
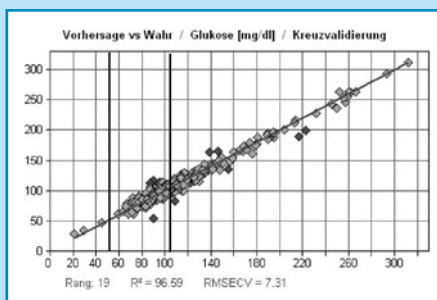
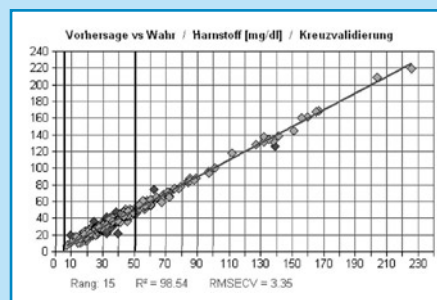


Fig. 1: Sampling system for the automated analysis of a large number of blood samples.

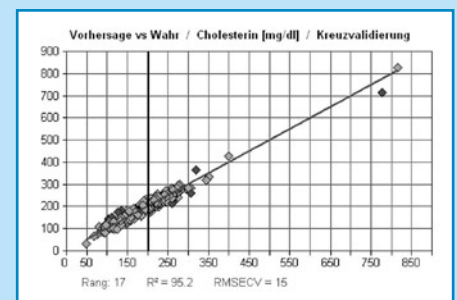
Fig. 2: Correlation diagrams for blood plasma samples. Vertical lines indicate clinically defined limits for normal parameters.



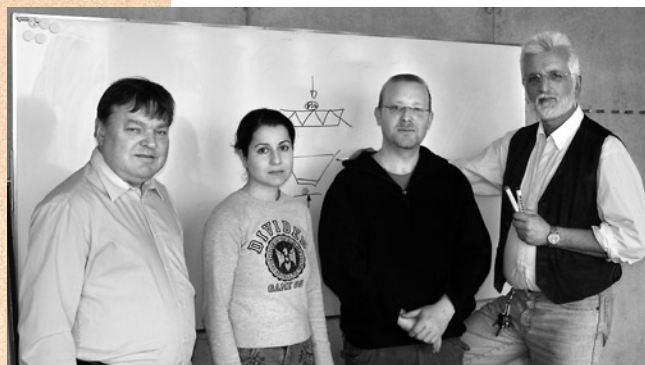
(a) Glucose



(b) Urea



(c) Cholesterol



From right to left: Prof. Dr. Werner Mäntele, Director of the Biophysics Institute; Dr. Oliver Klein, Staff member at the Institut für Biophysik; Dipl.-Phys. Gamze Hosafci, PhD student at the Institut für Biophysik; Prof. Dr. Gerhard M. Oremek, Head of Central Laboratory

range. They either use direct measurement of a pigment absorption or indirect measurement using a dye or enzyme reaction with subsequent spectroscopic detection. At present, detection and quantification in the mid-infrared spectral range from approx. 3–20 μm is not used in spite of the fact that the constituents of blood, urine or other body fluids exhibit highly specific fingerprint bands in this range due to their molecular structure. There are some attempts to use near-IR spectroscopy, however, with little success due to the lack of specificity. Some research groups have tried mid-IR analysis of dried samples of body fluids to overcome the problem of water absorption. The extra need of sample preparation such as a well-defined drying procedure and the risk of non-linearities, however, have up to now prevented clinical use.

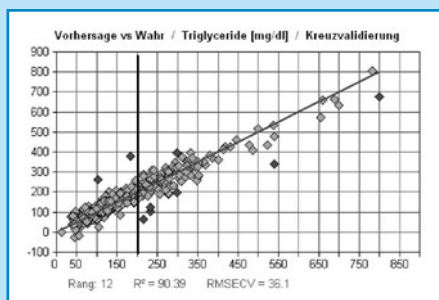
We have started some years ago to develop IR spectroscopic techniques for the analysis in clinical laboratories. Our goal was to develop a reagent-free method which can be easily used by nurses or even by untrained personal, which completely avoids the need for sample preparation, which requires only minute sample volumes, and which immediately provides the results, i.e. essential parameters, for the medical doctor. We have based our method on the optics of conventional Fourier transform infrared spectrometers and chosen attenuated total reflection

(ATR) as an optical interface for the measuring cells. Along this line, we have also been able to develop an analysis system for the quantitative determination of beverage ingredients (known as "beer sensor" and described in GIT/2005). For medical applications, miniature ATR cells have been developed where microsamples are either delivered manually or fed to the optical unit with an automatic sampling system (fig. 1). The ATR unit is integrated into the optical system of a compact FT-IR spectrophotometer. The sampling device is moved horizontally and vertically (see arrows) in order to bring individual samples subsequently in capillary contact to the ATR crystal and remove them after measurement. In addition, individual positions of the sampling device can be filled with a cleaning solution or with reference samples. The device resembles a microwell plate; the number of sample positions depends on the specific applications.

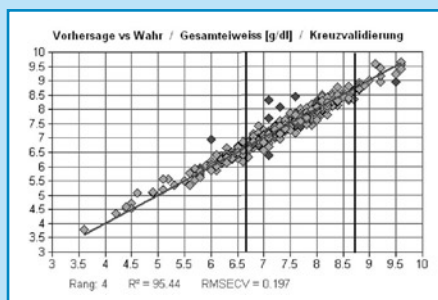
With this sampling system it takes less than a minute to record an IR spectrum of a liquid sample (full blood, serum, urine, etc.) which contains the entire information on the sample constituents. These liquids represent a complex matrix where different parameters can vary simultaneously and independent from each other. It is thus necessary to use specific multivariate analysis techniques like partial least square (PLS) or principal component regression (PCR) to analyze the spectra. With the procedures developed, we receive stable and reproducible component analysis.

Calibration of Reagent-free Analysis with Blood Samples

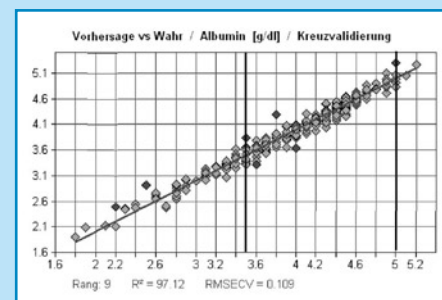
PLS- or PCR regressions require accurate calibration models. We have established such models from clinical full blood and urine samples selected carefully in order to cover the desired parameters in a range as large as possible. These samples thus cover the "normal" parameter values as well as pathological extremes, and, in addition, were analyzed in the clinical laboratory at best possible precision. This sort of careful "data design" is frequently neglected in multivariate analysis with the consequence that uncertain predictions for pathological parameter values



(d) Triglycerides



(e) Total protein



(f) Albumin

are made on the basis of an overweight of “normal” values. An estimation on the extent of calibration data needed for the prediction of six relevant parameters in their clinical variation leads to at least 100 carefully selected calibration samples before the precision of present analysis procedures is reached. We have purposely chosen a set of blood plasm samples as well as one of full blood samples (with EDTA added to prevent coagulation) in order to obtain calibration models for large-scale clinical analysis from plasm on one hand and for a direct and fast bedside measurement from full blood on the other side.

Lab-made Urine Samples: A Possibility for Calibration

For the analysis of urine samples a different procedure had to be chosen. Urine, in most clinical analyses, is only coarsely classified with respect to its content, for example as “compound not detectable”, “below xxx mg/ml”, or “above xxx mg/ml”. Setting up a calibration model on this basis is not possible. We have thus prepared artificial urine samples containing the most relevant compounds varying over several orders of magnitude. These calibration models were completed and validated with selected “real” urine samples. The composition of the artificial urine samples is shown in table 1.

Having thus established calibration models for full blood, blood plasm, and urine samples, arbitrary samples can be analyzed and predictions can be made from IR spectra. We present here the results of these analyses as correlation diagrams for blood plasm samples (fig. 2). For each sample, the concentration of the compound as obtained from established clinical chemistry analysis methods gives the abscissa value, while the ordinate value is the prediction from the IR spectroscopic analysis. To a large extent, the scattering of the data points is a result of the error of the established clinical method as observed with reference samples and by comparing the analysis of one sample from two independent measurements. An example for this scattering is the apparently low precision for triglyceride analysis, which, in the established analysis method, suffers from light scattering due to turbidity along with the enzymatic test used. Table 2 shows the range and the precision for our analysis of blood plasm and full blood EDTA samples. At present, we can reliably predict from these samples the concentration of glucose, urea, cholesterol, triglycerides, total protein, and albumin with a precision and reproducibility required for clinical application. Principally, further parameters can be predicted, however, at still insufficient correlation. Glucose presents a special case. The error for glucose determination, which for plasm samples corresponds to about that of test stripe reliability, increases to about 16 mg/dl for full blood samples. The reason for this decreased accuracy is glucose metabolism of blood cells due to a variable

time span in the hospital between taking the blood sample from the patient and the IR measurement. This was verified by adding small amounts of sodium fluoride, which inhibits glucose metabolism, to the full blood samples. With this procedure, the variable time between taking and analysis of the blood sample is not critical anymore, and the RMSECV value for full blood samples almost reaches that of plasm samples. Once the IR measurement is performed in a point-of care testing, and the patients blood is immediately analyzed, this procedure can be omitted.

The method reaches highest precision for urea – clearly a consequence of the relation between molecular structure and the relative dominance of the C=O bond in the IR spectra. For urea and triglycerides, the IR spectroscopic assay is only limited by the precision of the present clinical analysis which enters the calibration data set. On the other hand, the separate analysis of total protein and albumin at the precision obtained here is amazing: there are only minute spectral signatures that can be distinguished, and the concentrations for both components are covariant for most diseases. The reason for this success is a sufficient number of blood samples from patients with nephrotic syndrome in the calibration set. For patients suffering from this disease, the concentrations of total protein and of albumin in blood samples vary independently.

Table 3 summarizes the range and precision of the IR spectroscopic analysis of different components in urine. Urea, inorganic phosphorus, creatinine, ammonia, sulphur and ketone bodies can be determined at a precision which fulfills and partly exceeds clinical requirements. As a particular success, the IR spectroscopic analysis allows to further differentiate the total concentration of ketone bodies into hydroxybutyric acid and acetoacetate. For certain diseases such as diabetic ketoacidosis, where the ratio of both parameters is considerably altered, this represents a valuable additional information.

In addition to the components that can be quantitatively determined in their normal and

pathological concentration ranges, we have defined “threshold parameters” that can be registered from above a minimum concentration and that point to certain diseases. For example, a measurable threshold value for glucose in urine of 10–20 mg/dl points to an exceeded renal threshold and may be taken as a warning for diabetic patients. On the other hand, albumin in urine at a detectable value of 100–150 mg/dl is a clear hint for a macroalbuminuria. Along a line from simple urine test stripes to a full laboratory analysis, these results place the IR spectroscopic analysis close to the full laboratory analysis, however, at an effort as low as the use of a test stripe, not to speak about costs.

As a bottom line, we have succeeded in developing a simple-to-use, reagent-free analysis for blood, urine and other body fluids which, in contrast to established techniques, does not require sample preparation and procedures such as mixing, dilution, etc. It can be applied on site, i.e. next to the patient, and, in addition, considerably reduces potentially infectious waste. It is straightforward to couple this method with robotic devices for automated analysis. The sample volumes needed can be reduced to < 5 µl using miniature ATR devices. A full blood analysis for six blood parameters can thus be performed from a drop of blood in the same way a diabetic patient determines his glucose level, i.e. by pricking the fingertip. It can be transferred directly onto the optical interface. Cleaning of this interface is extremely simple.

On the other hand, the analysis described here can be easily combined with enrichment procedures in order to lower the detection limit or to screen for further blood components present at lower concentration. At present, detection limit and accuracy are based on calibration models which include several hundred selected samples. The research project will consequently be continued in a multi-center study in hospitals with thousands of samples. These samples will further improve the calibration models and further lead to higher reliability, selectivity and precision of this analysis method.

Table 1: Composition of artificial urine samples. Each compound was present at a low concentration limit, a high concentration limit, and at a “normal” value. For IR spectroscopy, these concentration series were combined in order to measure each component at the background of the variation of all other compounds.

Concentration (mg/dl)		low	average	high
Compound				
Urea		900	1900	3000
inorg. Phosphorous (as -PO ₄)		40	90	140
Creatinine		25	150	260
Ammonia (as NH ₄ Cl)		50	75	100
Sulphur (as Na ₂ SO ₄)		100	150	200
Protein (as Albumin)		0	1,5	3
Glucose		5	12	20
Uric acid		35	65	95
Ketone bodies	Hydroxybutyric acid	0	70	140
	Acetoacetate	0	70	140
	Aceton	0	10	20

Table 2: Range and precision (RMSECV) of blood parameters for blood plasma and full blood samples

	blood plasma [mg/dl]		EDTA-full blood [mg/dl]	
	range	RMSECV	Bereich	RMSECV
Glucose	20–350	± 7,31	20–340	± 16,7
Urea	5–230	± 3,35	10–220	± 4,5
Cholesterol	50–800	± 15	35–360	± 15,6
Triglycerides	0–800	± 36,1	0–1700	± 21,2
Total protein	3500–9800	± 197	2000–11000	± 319
Albumin	1600–5200	± 109	1000–6500	± 157

Perspectives

One one hand, direct application of this reagent-free analysis method in a clinical laboratory is evident. The automated analysis of large sample numbers promises a drastic cost reduction. In particular, this optical technique avoids regular calibration along with new reagent batches as well as dilution series. On the other hand, we can foresee a direct bedside application in care units, with the major advantage being the immediate availability of the data. The reliable determination of glucose also suggests applications in diabetes centers and care units for elderly people. With a suitable ATR unit, the continuous supervision of patients in dialysis units or in intense care units (ICU) is also possible.

Beyond this planned use, a number of further applications appear possible. As the analysis of urine can be easily made with a flow-through ATR cell, a combination of such a cell with a urinal is

suggested, which makes the determination of urine parameters as easy as determining weight or body fat – just a lot more precise than the latter.

Acknowledgments

The R&D project described here was performed at the Institut für Biophysik in close collaboration with the central laboratory of the University clinics. The authors would like Mrs. Sauer-Eppel for her help in the selection and analysis of the blood and urine samples. We are grateful to Prof. von Lilienfeld Toal (Main-Kinzig-Klinik, Gelnhausen) and Dr. Frithjof von Germar (formerly Elté GmbH) for numerous suggestions in the early phase of the project. The procedures and devices have been filed for patent by the Johann Wolfgang Goethe Universität Frankfurt am Main. The authors would like to thank Dr. Schöller (Innovectis GmbH) for his help and support during these steps.

Table 3: Range and precision for urine samples

	Range [mg/dl]	RMSECV [mg/dl]
Urea	900–3000	± 59,7
Inorganic phosphorous	40–140	± 4,33
Creatinine	25–260	± 11,7
Ammonia	50–100	± 2,93
Sulphur	100–200	± 4,4
Ketone bodies	0–300	± 20,4
- Hydroxybutyric acid	0–140	± 13,4
- Acetoacetate	0–140	± 7,58

► Contact

Prof. Dr. Werner Mäntele

Dr. Oliver Klein

Dipl.-Phys. Gamze Hosafci

Johann Wolfgang Goethe-University
Physics Department, Frankfurt am Main, Germany
Tel.: +49 69 798 46410
Fax: +49 69 798 46423
maentele@biophysik.uni-frankfurt.de
www.biophys.uni-frankfurt.de

Prof. Dr. Gerhard M. Oremek

University Hospital Frankfurt
Zentrum der Inneren Medizin, Frankfurt am Main, Germany
Tel.: +49 69 6301 5126
Fax: +49 69 6301 5498
www.klinik.uni-frankfurt.de/de/zentren/zim.asp