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Simultaneous determination of glucose, triglycerides, urea, cholesterol, albumin and total protein in human plasma by Fourier transform infrared spectroscopy: Direct clinical biochemistry without reagents

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ABSTRACT

Objective: Direct measurement of chemical constituents in complex biologic matrices without the use of analyte specific reagents could be a step forward toward the simplification of clinical biochemistry. Problems related to reagents such as production errors, improper handling, and lot-to-lot variations would be eliminated as well as errors occurring during assay execution.
We describe and validate a reagent free method for direct measurement of six analytes in human plasma based on Fourier-transform infrared spectroscopy (FTIR).

Design and methods: Blood plasma is analyzed without any sample preparation. FTIR spectrum of the raw plasma is recorded in a sampling cuvette specially designed for measurement of aqueous solutions. For each analyte, a mathematical calibration process is performed by a stepwise selection of wavelengths giving the optimal least-squares correlation between the measured FTIR signal and the analyte concentration measured by conventional clinical reference methods. The developed calibration algorithms are subsequently evaluated for their capability to predict the concentration of the six analytes in blinded patient samples.

Results: The correlation between the six FTIR methods and corresponding reference methods were $0.87 < R^2 < 1.00$. The interassay imprecision meets international quality criteria for all the six analytes. The linearity of the FTIR methods extends over the clinically significant concentration ranges. Visible hemolysis and icterus have some influence on the measurements. Plasma samples can be stored at 2–8 °C for at least 8 days before the analysis.

Conclusions: The developed FTIR methods use a simple and robust technology to achieve stable and accurate results that meet international quality criteria for the measurement of glucose, triglycerides, urea, cholesterol, albumin and total protein in human plasma.

Introduction

Clinical biochemistry of today is based on many years of research and development to provide valuable clinical information by precise, accurate, specific and sensitive analysis methods.

The routine methods of today must be simple, rapid, robust and suited for automation to meet the ever increasing demand for efficiency and high sample throughput in the clinical laboratory.

Most clinical analyses follow the same pattern. A patient sample containing the analyte is mixed and incubated with a reagent, and a chemical reaction then takes place between the analyte and the reagent molecules. The chemical reaction is selected to give product molecules with characteristic physical properties, most often a specific chromophore. The amount of product molecules produced by the reaction is measured as an absorbance change, at a specific wavelength, and by means of a calibration curve used to calculate the analyte concentration.

For each component to be measured, a specific reagent is required. Thus, in a clinical chemistry laboratory, a large number of reagents must be stored under controlled conditions and prepared carefully according to specific procedures, to achieve and maintain optimal analysis quality. Analytical errors are often caused by improper handling of reagents or by lot-to-lot variation of the reagents.

Methods without the use of reagents have been applied in clinical laboratories for many years. There are many obvious advantages of these methods. Reagent expenses as well as time-consuming handling procedures such as purchasing, storage, reconstitution and inventory

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control are avoided, and analytical errors caused by the reagents and errors occurring during the assay execution are eliminated.

Atomic absorption spectroscopy, flame emission spectroscopy, UV/vis spectroscopy and ion selective potentiometry are examples of reagent free methods applied on biological samples. In atomic absorption spectroscopy and flame emission spectroscopy, the biological sample is combusted and the absorbance or emission at specific wavelengths is used to determine the concentration of metallic ions. Ion selective electrodes have been developed to measure the free concentration of some ions (Na^+, K^+, Ca^{2+}, Li^+,Cl^-) by a direct contact between the biological sample and the ion selective electrode. Some biomolecules with characteristic UV/vis absorbances (hemoglobin and bilirubin) have been determined in both serum and cerebrospinal fluid by direct UV/vis spectroscopy [1,2]. In this case multiple wavelengths are used to estimate oxyhemoglobin, methemoglobin and bilirubin at the same time. All these methods are known to be stable and robust, when the measuring instruments are properly maintained.

In recent decades, reagent free methods based on the analysis of more complex spectra have been developed. Nuclear magnetic resonance spectroscopy (NMR) has been employed to investigate metabolic disorders in human diseases [3,4]. NMR technology requires relatively little sample preparation, and no purification or separation steps. NMR can be used to perform absolute quantification of abundant low molecular weight metabolites, but the technique is characterized by a relatively low analytical sensitivity and high data complexity [5].

Mass spectrometric methods (MS) have a much higher analytical sensitivity. With MS it is possible to perform absolute quantification of hundreds of low-concentration components in human plasma. Biological samples may be injected directly into the MS, but almost all published MS methods employ a pretreatment of the sample and a chromatographic step to improve the specificity and signal to noise ratio of the MS method. Although mass spectrometry may be characterized as a direct, reagent free method, a lot of solvents and isotope-labeled standards are usually required as well as highly trained technicians to operate the MS instrument.

Direct methods based on near-infrared (NIR) spectra [6–8] or mid-infrared (IR) spectra have also been developed. These spectra are rather complex, but by the application of chemometric methods, attempts have been made to estimate a number of clinically significant components at the same time in human serum and other physiological fluids. Attenuated total reflection (ATR) is an easily manageable technique to record IR spectra. An attempt was made using this technique, combined with chemometric data processing, to develop a point-of-care method for the determination of a number of clinical components in serum [9]. The authors conclude that albumin and total protein could be determined successfully, while glucose, urea, HDL, LDL and cholesterol were predicted with relative errors between 15 and 23%, which is only sufficient for application as a screening tool.

The best results so far have been obtained by Fourier-transform mid-infrared transmission spectroscopy [10–12]. In these methods a well defined volume of serum or urine is mixed with an internal standard, and dried down to a thin film to eliminate the spectral interference of water. This rather laborious and time consuming sample preparation makes these methods unsuited for acute analysis in the clinical laboratory.

Here we present a method for simultaneous determination of glucose, triglycerides, urea, cholesterol, albumin and total protein in unmodified human plasma by Fourier-transform mid-infrared transmission spectrometry and chemometry. No pre-treatment of blood plasma is needed before analysis. The total time of sample measurement and calculation of the concentration of all the six analytes is about 2–3 min. The simplicity and robustness of the method and the short analysis time open the possibility for an application of this technique in the clinical laboratory.

Materials and methods

Sample collection

The study was approved by the research ethics committee.

Patient samples were obtained by venipuncture and collected in Li-heparinate gel separation tubes (Venosafe, VF-054SAHL). Plasma was, within one hour, separated from the cellular fraction by centrifugation at 1200 *g* for 10 min, and analyzed within 3 h, or plasma was frozen at −20 °C for later analysis.

FTIR measurement procedure

FTIR spectra were recorded with a Tensor 27 FTIR spectrometer from Bruker Optics, Ettlingen, Germany, with a nitrogen cooled mercury cadmium telluride detector. Transmission was measured in a semi-automated flow-through unit (MIU100) from micro-biolytics GmbH Esslingen, with an integrated 25 °C water-thermostated CaF cell (AquaSpec eCell AS1100). The cell is specially designed for the measurement of aqueous solutions. An optical path length of only 8.5 μm and a solid cell structure provides stable measurement with a relatively low water background. The samples (40 μL) are applied manually into the measuring cell, and between each sample loading the cell is flushed automatically with sterile water for about 30 s. Injection port filters ensure that no particles contaminate or clot the cell during the filling process. Serum or heparin plasma can be used as sample, but EDTA plasma must be avoided due to the calcium chelating properties of EDTA, which will slowly degrade the CaF of the measuring cell.

For each sample 128 interferrograms were co-added to yield spectra with a nominal resolution of 4 cm^{-1}. The spectra were recorded in the spectral range between 3100 and 950 cm^{-1}. A zero filling factor of 4 was employed, yielding point spacing in the spectra of 1 cm^{-1}. In the analysis the first derivative of the original spectra was used. The first derivative was computed by using the Savitzky–Golay algorithm with 9 smoothing points. For each sample two IR spectra were recorded and the mean spectrum was subsequently corrected by the subtraction of a water blank spectrum.

Clinical reference methods

Measurements by reference methods were carried out at the Department of Clinical Biochemistry at Holbaek University Hospital on Cobas C 501 autoanalyzer (Roche Diagnostics D-68298 Mannheim) with reagents supplied by the manufacturer. Glucose was measured by a hexokinase method [14] (Catalog nr. 04404483 190, CV% < 1.5%), triglycerides by enzymatic colorimetry [15] (Catalog nr. 20767107 322, CV% < 2.0%), urea by enzymatic photometry [16] (Catalog nr. 04460715 190, CV% < 1.4%), cholesterol by enzymatic colorimetry [17] (Catalog nr. 03039773 190, CV% < 1.7%), and total protein by biuret method [18] (Catalog nr. 03183734 190, CV% < 2.5%). Albumin was measured by immuno-turbidimetry (Catalog nr. 04469658 190, CV% < 2.2%) or by colorimetric bromocresol green method (BCG) (Catalog nr. 04657357 190, CV% < 1.8%). All procedures recommended by the manufacturer were followed.

Calibration of FTIR methods

Calibration of the FTIR methods was carried out with 144–169 patient samples, selected to cover as large a concentration range as possible. Most of the patient samples were used for the calibration of all six analyses. The general calibration process is described in detail by Höskuldsson [13].

Many separate steps constitute the calibration process. For each analyte the FTIR spectra are processed by a stepwise selection of wavenumbers giving the optimal least-squares correlation between the measured IR signal, and the analyte concentration measured by the reference method. Wavenumbers showing no or small correlation

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