The dissociation constants ($K_D$) of the BsF$_{\mu}$ and its parental F(ab')$_{2\mu}$ fragments with their specific antigens were measured by surface plasmon resonance using a BIAcore biosensor (Pharmacia Biosensor). The BsF$_{\mu}$ and its parental F(ab')$_{2\mu}$ fragments were injected separately and immobilized on the chip coated with goat anti-mouse (IgG + IgM) antibodies (Jackson ImmunoResearch Laboratories). When CEA (50 mg/L; Scripps Laboratories) was injected on the chips coated with BsF$_{\mu}$ or its parental F(ab')$_{2\mu}$ fragments, the intensities increased 29 and 30 relative response units, respectively. The intensities of BsF$_{\mu}$ (50 mg/L) and its parental F(ab')$_{2\mu}$ fragments (50 mg/L) against AP increased 23 and 24 relative response units, respectively. For CEA, the $K_{dS}$ were 249 nmol/L (BsF$_{\mu}$) and 212 nmol/L [F(ab')$_{2\mu}$ (9CA10)], respectively. The $K_{dS}$ against AP were 13 nmol/L (BsF$_{\mu}$) and 10 nmol/L [F(ab')$_{2\mu}$ (APM05)], respectively.

The immunoreactivity of BsF$_{\mu}$ bound to AP was evaluated by sandwich enzyme immunoassay and compared with that of the F(ab')$_{2\mu}$ covalently conjugated to AP. A 96-well microtiter plate (Nunc-intermed; Maxisorp) was coated with goat anti-CEA polyclonal antibody (prepared in our laboratory) and blocked by incubation with 2 g/L bovine serum albumin in PBS (pH 7.4). After washing, the plate was incubated at 25 °C for 60 min with 100 μL of CEA at various concentrations (0, 25, 50, 100, 200, 400 μg/L) and 100 μL of the BsF$_{\mu}$ bound to AP (absorbance at 280 nm in PBS = 0.004), IgM covalently labeled with AP (absorbance at 280 nm in PBS = 0.002), or (F(ab')$_{2\mu}$ covalently labeled with AP ($A_{280\text{ nm}}$ in PBS = 0.004). After another washing, 100 μL of the substrate solution (2 g/L p-nitrophenylphosphate in 50 mmol/L carbonate buffer, pH 9.5, containing 10 mmol/L MgCl$_2$) was added to the plate for 20 min at 25 °C, and the reaction was terminated by adding 100 μL of 0.5 mol/L NaOH. The AP activity was measured by the absorbance at 405 nm (Fig. 1B). The detection limits of the CEA assay (defined as 2 SD above the zero concentration calibration) were 0.90 μg/L for BsF$_{\mu}$ and 3.4 μg/L for F(ab')$_{2\mu}$, respectively. The immunoreactivity of BsF$_{\mu}$ against CEA was almost the same as that of the F(ab')$_{2\mu}$ covalently labeled with AP in the range 0.90–400 μg/L. The nonspecific binding of BsF$_{\mu}$ was 27% ((0.90 × 100)/3.4 = 27) lower than that of F(ab')$_{2\mu}$.

Because the covalently labeled conjugate that was prepared from F(ab')$_{2\mu}$ and AP is a complex polymer molecule and has multiple antibody binding sites in one conjugate molecule, the conjugate cannot bind to the antigen at a molar ratio of 1:1. The BsF$_{\mu}$ bound to the conjugate can bind more efficiently to the antigen at a 1:1 molar ratio. To detect lower concentrations of the antigen, BsF$_{\mu}$ may be more useful than the covalently labeled conjugate. The BsF$_{\mu}$ bound to the AP conjugate was stable for at least 3 months at 4 °C.

In conclusion, we showed that homogeneous BsF$_{\mu}$ fragments prepared from IgMs recognized both antigens without any loss in immunoreactivity. The BsF$_{\mu}$ bound to AP allowed sensitive and reliable measurement of the antigen and may be generally applicable for immunodiagnosis.

### References


### Toward Reagent-free Clinical Analysis: Quantitation of Urine Urea, Creatinine, and Total Protein from the Mid-Infrared Spectra of Dried Urine Films, R. Anthony Shaw,1* Sarah Low-Ying,1 Michael Leroux,2 and Henry H. Mantsch1

Infrared (IR) spectroscopy offers an approach to clinical analysis that is conceptually very appealing. Whereas countless assays rely on the use of chemical agents to “recognize” the analyte of interest and to react with the analyte to produce specific color changes, IR-based analysis is founded on the rich IR absorption patterns that characterize the analytes themselves. These absorption patterns provide the basis to distinguish among the chemical constituents and to separately quantify them. The most obvious distinguishing feature is that no reagents are required. In addition, IR-based analytical methods require very small sample volumes (typically microliters), show good precision over the entire physiological range, and are well suited for automation.

Several previous studies have illustrated potential roles for IR spectroscopy in the clinical laboratory. For example,
six serum analytes have been shown to be suitable for IR-based analysis, namely albumin, total protein, glucose, triglycerides, urea, and cholesterol (1–6). Studies of amniotic fluid have yielded IR models to quantify the lecithin/sphingomyelin ratio and the surfactant/albumin ratio, establishing IR spectroscopy as an attractive option for the assessment of fetal lung maturity (7, 8).

There are several approaches to IR-based analysis, with the first choice being whether to use the near-IR (750–2500 nm) or mid-IR (2.5–100 μm) spectral range. Near-IR spectroscopy has gained notoriety within the clinical chemistry community through the many efforts to develop a noninvasive blood glucose monitor based on this technology [see e.g., Refs. (9, 10)], and in that vein it has been shown that glucose concentrations can be recovered from the near-IR spectrum of native serum (3).

The main reason for the focus on near-IR spectroscopy is that tissue is quite transparent to near-IR light, hence the attraction for in vivo work. However, this is obviously not a factor for in vitro analysis. The mid-IR spectrum offers some potential advantages. Near-IR spectroscopy typically requires a sample volume of at least 0.1–0.2 mL, whereas a mid-IR assay can be carried out with ≤10 μL. Although water contributes enormous absorption bands in the mid-IR, these can be eliminated by simply drying the sample to a film and using the spectrum of the dry film as the basis for analysis (6–8). This film may then be archived for subsequent reanalysis.

The present study was conducted to evaluate the sensitivity and accuracy of mid-IR spectroscopy in the determination of urine urea, creatinine, and total protein. The IR-based quantification methods were calibrated by comparison with the results provided by standard clinical chemistry assays. To that end, urea [enzymatic (urease) conductivity], creatinine (Jaffe rate), and total protein (benzethonium chloride reaction) concentrations were determined for 200 urine samples. Urea concentrations were 40–440 mmol/L, creatinine concentrations were 1.5–18 mmol/L, and total protein was 0.02–20 g/L. Samples were prepared for IR spectroscopy by first adding 0.1 mL of aqueous (4 g/L) potassium thiocyanate solution to 0.5 mL of the urine sample. Duplicate films were prepared by drying 12 μL of this mixture onto IR-transparent BaF2 substrates, and mid-IR absorption spectra were acquired at ambient temperature for the dry films (Bio-Rad FTS-40A Fourier transform IR spectrometer operating at 4 cm⁻¹ resolution, with 512 scans averaged for both the sample and background spectra). An isolated thiocyanate absorption at 2060 cm⁻¹ then provided the basis to normalize all spectra to a common effective optical pathlength.

Quantification methods were derived by using partial least-squares regression (PLS) to establish relationships between the IR spectra and the reference analyses. A training set of 133 specimens (266 spectra) was used to calibrate quantification methods for each of the three analytes. The test set, comprising the remaining 67 specimens (134 spectra), served to test the validity of the IR-based assays. The accuracy of the PLS quantification models was improved by using spectral subregions rather than the entire 800-5000 cm⁻¹ range that was available. The appropriate spectral regions for PLS were determined by first carrying out a series of exploratory trials using limited spectral ranges and fine-tuning those ranges based on the standard errors in the training and test sets. The number of PLS factors in the final model was set at 1 for urea (7 factors), 11 for creatinine (11 factors), and 16 for total protein (16 PLS factors), 1400–1800 cm⁻¹ for creatinine (11 factors), and 3100–3550 cm⁻¹ for urea (7 factors). Scatterplots comparing the IR-predicted protein, creatinine, and urea concen-

---

![Fig. 1. Scatterplots comparing IR-predicted urea (top), creatinine (bottom), and protein (middle) concentrations with the concentrations provided by accepted clinical analytical methods.](https://academic.oup.com/clinchem/article-abstract/46/9/1493/5641252)

Regression lines (y = ax + b, where y is the IR-based analysis, and x is the reference analysis): for creatinine, a = 0.99, b = 0.03 mmol/L, r = 0.98; for total protein, a = 0.97, b = 0.08 g/L, r = 0.94.
trations to the reference analyses for this set of test specimens are shown in Fig. 1. The IR-based analytical methods yielded creatinine concentrations with a SD$_{dup}$ [the root mean square difference between IR-predicted and reference analyte concentrations for the test set only] of 0.58 mmol/L ($r = 0.98$) for creatinine, 14.1 mmol/L ($r = 0.98$) for urea, and 0.48 g/L ($r = 0.94$) for protein.

The distribution of protein concentrations is skewed heavily, with the majority of specimens showing concentrations well below 1 g/L (Fig. 1, middle panel). As a result, the best approach to IR-based protein quantification is to use two models rather than one. A second PLS quantification model was optimized for those samples with concentrations <1 g/L, yielding SD$_{p}$ = 0.13 g/L, although this still falls short of the performance required for accurate quantification at typical low protein concentrations, the method is sufficiently accurate to serve as a coarse screening test.

The ultimate accuracy of the IR-based methods is influenced in part by the accuracy of the reference methods used to calibrate them. This is not a factor for the protein analysis, where the reference method is clearly more accurate than the IR-based method, but it may play a role for both urea and creatinine. This possibility is suggested by the precision of the IR-based assays: SD$_{dup}$ = 0.18 mmol/L for creatinine, 6.8 mmol/L for urea, 0.14 g/L for protein (including all samples), and 0.05 g/L for protein concentrations <1 g/L.

At least part of the gap between the precision and accuracy of the urea (SD$_{u}$ = 14.5; SD$_{dup}$ = 6.8 mmol/L) and creatinine (SD$_{p}$ = 0.54; SD$_{dup}$ = 0.18 mmol/L) assays may be attributable to scatter in the reference methods themselves.

The mid-IR quantification methods presented here match or exceed the performance of the near-IR methods presented previously (12). Both approaches yield analyses that are accurate enough to serve as a routine method for urine urea and creatinine analyses. Although protein concentrations are too low for accurate quantification using IR spectroscopy, the method may serve as a screen to detect concentrations above ~0.5 g/L and to quantify at those concentrations.

The practical implementation of this and other clinical IR-based assays requires two key developments. One of these is the discovery of an inexpensive substrate to substitute for the costly BaF$_2$ windows that were used as part of this work. Although these windows can be cleaned and used repeatedly, this is probably impractical in high-volume laboratories. A surprising alternative has emerged recently, as we have shown recently that many analyses can be carried out using ordinary glass as the substrate, despite its limited transparency in the mid-IR region (13, 14). The stumbling block that remains in place is a practical one, that being automation of the method. The practical benefits of IR-based methods are being realized in an extraordinary range of analytical applications (15), and it would seem to be only a matter of time before these methods find their way into the clinical realm.

References