necessary microscopic checks of flagged samples from 142 to 11 samples.

In this study, the localization of hematuria was based on the clinical diagnosis and morphological observations. These classifications were compared to the morphology flags obtained in the UF-100. The UF-100 has fixed algorithms for morphology flagging. In our study, the UF-100 correctly classified 37 of 53 postrenal (70%) and 66 of 86 renal hematurias (77%). In 20 cases, the UF-100 could not correctly classify hematuria because of low RBC counts.

These findings indicate that the combination of test strips with UF-100 flow cytometry can reduce and practically eliminate false-negative or -positive results that are obtained (−2%); these samples can be reviewed microscopically. The major reason for discrepant results was misclassification of particles (yeast or bacteria for lysed RBC). Scrutiny of the scattergrams, however, is crucial for correct classification of these particles. In addition to improved accuracy, our strategy yields quantitative results for particular elements in urine. This may be an improvement to the microscopic counts currently used for monitoring of renal diseases.

Because microscopic review of urine specimens requires 6–8 min per specimen, our approach reduces manual labor, and the majority of specimens can be analyzed using automated techniques. Furthermore, the reliability is improved by automation. The UF-100 displays several flags for microscopic review. In most cases, these reviews can be circumvented by using test strips in parallel and diluting samples with high total cell counts. However, the manufacturer-defined review flags for SRCs and pathological casts can be confirmed accurately only by microscopy. In our opinion, these findings are of less diagnostic importance because quantitative urinary protein determination offers higher diagnostic reliability in renal disease.

Renal and postrenal hematuria can be distinguished relatively well based on the isomorphism or dysmorphism flags of the UF-100. However, delay in analysis because of transportation problems combined with low RBC counts may also be a critical factor.

In summary, the combined sequential analysis of urine sample with a test strip analyzer and the UF-100 flow cytometer appears to be better than the standard procedures used to date. The main advantages are that the majority of specimens can be analyzed automatically, thus reducing manual labor and turnaround times. In specific cases, however, special microscopic techniques such as sediment analysis with or without supravital staining can still be used as auxiliary techniques.

References

Rapid and Sensitive Immunoassay for the Measurement of Serum S100B Using Isoform-specific Monoclonal Antibody, Miyoko Takahashi, Andrea Chanczuk, Yuwen Hong, and George Jackowski (Skye PharmaTech, Inc., 6354 Viscount Rd., Mississauga, ON L4V 1H3, Canada; * author for correspondence: fax 905-677-1674, e-mail empty@ICA.net)

S100 is an acidic calcium-binding protein with a molecular weight of 21 000, originally discovered by Moore (1) in the bovine brain. Today, >14 different S100 members are known (2), of which S100A1 and S100B are the most studied (3–6). Because a high concentration of S100B is present in the brain, S100B has been studied for use as a biochemical marker for central nervous system pathology. Numerous studies in the literature have suggested the clinical usefulness of measuring this protein in cerebrospinal fluid (7–11), and in recent years, serum S100B has been reported as a useful marker for early detection of metastases of melanoma and cerebral complications from head injury, cardiac surgery, and acute stroke (12–21).

With the advent of therapeutic treatments for stroke that either dissolve the clot or protect the brain, early diagnosis of stroke and the identification of appropriate patients for intervention are increasingly important. Existing assays lack sensitivity and ease of use and usually require ≥3 h to perform. We have, therefore, developed an ELISA that is rapid, highly sensitive, and S100B specific. This ELISA was used to retrospectively assess S100B concentrations in the serum of stroke patients.

Human S100B cDNA (Genome System), amplified by PCR, was cloned into vector pET28a (Novagen) and expressed in Escherichia coli BL-21 (DE3)pLysS (Novagen). Recombinant human S100B (rS100B) was isolated from host cells that were induced with 1 mmol/L isopropyl-β-D-galactoside for 2 h at 37 °C and were lysed with a native buffer system. S100B was purified with an Ni-NTA affinity column.

The ELISA buffers were as follows: plate-coating buffer (100 mmol/L carbonate buffer, pH 9.6); phosphate-buffered saline (PBS; 1.5 mmol/L KH2PO4, 8.5 mmol/L Na2HPO4, 2 mmol/L KH2O, 2.7 mmol/L KCl, 137 mmol/L NaCl, pH 7.4); washing buffer (PBS containing 0.5 g/L Tween 20).
20, pH 7.4); blocking buffer (10 mmol/L Tris, 10 g/L hydrolyzed casein, 5 g/L bovine serum albumin, pH 8.0); incubation buffer (PBS containing additional NaCl to a final concentration of 300 mmol/L and 1 mmol/L calcium lactate); calibrator diluent (PBS containing 500 mL/L normal human serum, pH 7.4); detector antibody dilution buffer [PBS containing 1 g/L bovine serum albumin, 1 mmol/L K$_2$Fe(CN)$_6$·4H$_2$O pH 7.4]; and conjugate antibody dilution buffer [PBS containing 100 mL/L heat-inactivated fetal bovine serum and 1 mmol/L K$_2$Fe(CN)$_6$·4H$_2$O pH 7.4].

Female BALB/c mice (7–8 weeks of age) were immunized by subcutaneous injection of 30 µg of bovine S100B emulsified in complete Freund’s adjuvant. Intrapерitoneal booster injections were given 4 weeks later (30 µg in incomplete Freund’s adjuvant) and then at 3-week intervals (three times or more), with a final injection of 50 µg of antigen in buffer via intravenous/intrapерitoneal routes 3 days before cell fusion. Hybridoma cultures were screened by ELISA, and positive cultures were cloned at least twice with limiting dilutions as described by Fuller et al. (22). The monoclonal antibodies (MAbs) were purified using protein G chromatography (Pharmacia Biotech), and their subclasses were determined (Mouse Typer®, Bio-Rad).

The specificities of purified MAbs were first assessed on microtiter plates coated with S100B-B (ββ), S100B-A1 (αβ), and S100A1-A1 (αα) and blocked with blocking buffer; the S100 proteins from bovine brain were from Sigma or Affinity Research Products. MAbs that showed no reactivity with S100A1-A1 were further characterized in a sandwich ELISA (see below). We also tested for cross-reactivity with myelin basic protein (from Dr. M.A. Moscarello, University of Toronto, Toronto, Canada), neuron-specific enolase (yγ and αα-isofoms; from Dr. M.D. Coughlin, McMaster University, Hamilton, Canada), calmodulin (Sigma), and synapsin-1 (Skye PharmaTech). The reaction rate constant, the binding affinities of the MAbs for recombinant S100B, and the optimal MAb pair for ELISA were determined on a BIAcore instrument (Pharmacia).

MaxiSorp™ plates (Nunc) were coated for 18 h at room temperature (19–23 °C) with 1 mg/L MAb S27 in 0.125 µL of coating buffer per well. The plates were then washed once, and the free binding sites were blocked with 0.25 mL of blocking buffer at room temperature for 1 h, after which the plates were washed three times. Recombinant S100B was used for calibrators. The purified rS100B was quantified by the Bradford method (Bio-Rad). The rS100B stock solution was diluted to a concentration of 10 mg/L in the calibrator diluent and kept frozen in 100-µL aliquots at −75 °C until use. After thawing, the rS100B solution was further diluted to 0–3.2 µg/L in the diluent supplemented immediately before use with 4 mmol/L Pefabloc SC and PSC-protector (Boehringer Mannheim) and 1 mL/L ProClin-300 (Supelco), followed by filtration through a 0.2 µm membrane.

All the ELISA incubation steps were carried out at room temperature on an orbital shaker unless otherwise stated. Calibrators and serum samples were pipetted in duplicate into the microtiter plate wells (25 µL/well), followed by the addition of 75 µL of incubation buffer to each well. After 15 min of incubation, the plate was washed three times, 100 µL of detector antibody (rabbit anti-bovine S100 (Dako)) was added, and the plate was incubated for 15 min. The plate was again washed three times, followed by an addition of peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratory) at 100 µL/well. The plate was incubated for 10 min. After washing, the bound enzyme activity was visualized by the addition of 100 µL of 3,3′,5,5′-tetrachlorotetraethylbenzidine substrate solution (Sigma). The substrate hydrolysis continued for 5 min at room temperature in the dark, at which point the reaction was stopped with 100 µL of 0.5 mol/L H$_2$SO$_4$. Absorbance was measured in a microplate reader (Bio-Rad) at 450 nm. The Bio-Rad microplate reader program calculated the S100B concentrations of the unknown samples by reference to a quadratic calibration curve.

From three cell fusions, 14 clones were established: 2 clones were IgMk and 12 clones were IgG1,k. All clones were specific for S100B and did not cross-react with S100A1, neuron-specific enolase-γγ, neuron-specific enolase-αα, myelin basic protein, and synapsin-1. However, all clones showed ~0.1% cross-reactivity with calmodulin at 10 mg/L. Interestingly, epitope studies on BIAcore indicated that all 12 IgG clones recognized the same epitope. The commercially available MAb, SH-B1 (Sigma), also recognized this epitope. The clone S27 was selected for our assay development on the basis of its S100B specificity determined by ELISA (Fig. 1A) and its high affinity as determined by BIAcore. A two-site ELISA was developed using MAb S27 as capture antibody, to make the assay S100B specific, and polyclonal rabbit anti-S100 as detector. The total assay time was 45 min, with a detection limit for S100B of 0.0125 µg/L (zero calibrator + 2 SD; n = 20). The calibration curve was from 0 to 3.2 µg/L. The imprecision (CV) was assessed by the measurement of three serum samples supplemented with rS100B at concentration of 0.2, 0.8, and 3.2 µg/L in 20 consecutive analytical runs for interassay precision, and 20 determinations of the same concentrations using a pool of the three sera for intraassay precision. The maximum CV observed was 6.7%. No hook effect was observed at a S100B concentration of 200 µg/L, although the ELISA signal reached its maximum at ~50 µg/L.

All serum or plasma samples obtained were aliquoted into plastic vials within 1 h of blood collection and were stored at −75 °C until use. The serum concentrations of S100B were determined in 103 healthy subjects to establish the lower limit of detection. All stroke samples were collected from patients admitted within 24 h of symptom onset. Blood samples were collected on day 1 (at presentation), and on days 2, 3, and 7 at one hospital and on days 1, 2, and 3 at a second hospital.

To assess analytical recovery, we added 0.2 and 0.8 µg/L rS100B to three human serum samples. The measured values were 91–108% (mean, 97.8%) of the expected values. To examine the linearity of the assay, four patient
Fig. 1. Binding curves for MAb S27 with adsorbed S100 isoforms (A) and serum S100B in stroke patients (B).

(A), α, α isoform; ○, αβ isoform; ●, ββ isoform. (B), S100B (μg/L) was measured in serum collected upon presentation (day 1) and 24 h later (day 2). Horizontal line in the left panel indicates the cutoff value (0.021 μg/L). Panel on the right (y-axis, full scale = 0.25 μg/L) is an expansion of the lower part of the panel on the left (y-axis, full scale = 3.5 μg/L).
sera with S100B >1 µg/L were serially diluted with the zero calibrator (2×, 4×, 8×, and 16-fold dilutions) and measured in triplicate. The ratio of measured values to expected values was 85–110%, indicating a lack of interference by serum components with the assay. The effect of potentially interfering substances (bilirubin, hemoglobin, and lipids) in the S100B assay was also determined in specimens containing 3.2 µg/L S100B. The presence of these substances had no significant effect on assay performance. To examine the effect of anticoagulants in the assay, we added rS100B (3.2 µg/L) to matched serum and plasma samples from six blood donors and measured the S100B in triplicate. The values in heparinized plasma were 93–151% of the serum values. With citrate or EDTA, values were lower (20–46% of serum values for EDTA plasma and 44–78% for citrate plasma). The values for S100B from citrated plasma became equivalent to serum values when 5 mMmool/L Ca²⁺ was added to the incubation buffer.

The mean S100B in sera from 103 healthy donors (age range, 18–78 years; mean age, 54.6 years; approximately equal numbers of males and females) was 0.0067 µg/L with a 98th percentile of 0.021 µg/L. Serum S100B in healthy subjects showed no relationship with age or sex, contrary to previous reports (23, 24). The published reference ranges for S100 in cerebrospinal fluid differ considerably, from <0.51 µg/L by Mokuno et al. (10) to <1–6.8 µg/L by Persson et al. (11). Comparing S100 results from the various studies is complicated by the authors’ use of different antibody pairs and assay protocols, particularly when different sample matrices are involved. The specificity of other anti-S100B antibodies may also contribute to the variability of the previous results.

All patients with stroke admitted within 24 h of symptom onset were included. The mean age of the stroke patients was 66 years, with an age range of 27–90 years. Of the 32 stroke patients, 26 had ischemic strokes (81%), 5 of which were lacunar types (16%); 4 patients had hemorrhagic stroke (13%), of which 3 had subarachnoid hemorrhage and 1 had an intracerebral bleed; and 2 patients had transient ischemic attacks (6%). At presentation, S100B was >0.021 µg/L in 44% of the patients. Two days later S100B was >0.021 µg/L in 74% of the patients (Fig. 1B). Subsequent serum samples had either increased or decreased concentrations of S100B, presumably depending on whether the stroke was evolving in severity or subsiding. Seventeen of 32 patients (53%) had a computed tomography (CT) scan performed upon presentation. Of the 17 patients, 9 were positive (53%) by CT on presentation. All four hemorrhagic patients were CT and S100B positive at presentation. Eight of the 17 patients (47%) had a normal CT scans at presentation (which became positive days later). Two of the eight CT-negative patients (25%) were positive for S100B. In six patients with lacunar circulation infarctions and four with transient ischemic attacks, S100B was not increased at presentation.

Therapeutic considerations make the 45-min turnaround time for results in this assay an important factor, particularly because the only other commercially available S100 assay requires more than twice as much time to perform. The value of biochemical markers for the diagnosis of any disease depends on the time frame available for effective treatment. The clinical utility of rapid S100 testing is still being established. Previous reports described increased S100 in cerebrospinal fluid-serum collected from stroke patients days rather than hours after the onset of stroke (11, 12, 25, 26). In contrast, the ELISA described here detected increases in serum S100B in 44% of the patients at presentation.

The current gold standard for making the diagnosis of stroke is CT scanning in addition to neurological examination (27). Although only 44% of the stroke patients showed increased S100B at presentation, an encouraging result was that increased concentrations were detected in two of eight CT-negative patients. The latter data provide an impetus for the continued evaluation of S100B as a useful biochemical marker for stroke diagnosis.

We thank Drs. R. Jaeschke, E. Stanton, and M. Lawrence of St. Joseph’s Hospital and Dr. M. Hill of St. Michael’s Hospital for the supply of stroke serum samples. We also thank Dr. Roger MacKenzie of National Research Council Canada for BiAcore analysis of our monoclonal antibodies.

References