Validation and Comparison of Two Solid-Phase Immunnoassays for the Quantification of S-100B in Human Blood, Ulrich Misslere, Martin Wiesmann, Philipp Ehlermann, Michael Tronnier, Axel Nötzold, Elke Steinmeier, and W. Graham Wood (1 Institute of Radiology, 2 Department of Cardiology, 3 Department of Dermatology, and 4 Department of Cardiac Surgery, University of Luebeck Medical School, 23538 Luebeck, Germany; 5 Institute of Clinical Chemistry, Municipal Hospital, 18435 Stralsund, Germany; * address correspondence to this author at: Institut fuer Radiologie, Medizinische Universitaet zu Luebeck, Ratzeburger Allee 160, 23538 Luebeck, Germany; fax 49-451-500-6190, e-mail missler@medinf.mu-luebeck.de)

S-100 protein concentrations in serum are considered a quantitative marker of the extent of damage to the central nervous system (CNS) (1–3), including possible cerebral injury following procedures such as coronary artery bypass surgery (4, 5). There are 19 S-100 proteins, of which S-100A1 and S-100B are the most prevalent (6). S-100A1 and S-100B form dimeric proteins that previously were labeled S-100a (S-100A1-B), S-100b (S-100B-B), and S-100a0 (S-100A1-A1) (7–9). Initial studies of S-100 protein reported that this protein is present only in the CNS (7), but later studies found various concentrations of S-100A1 and S-100B in tissues outside the CNS, including the heart and aorta (10). At present, there are questions about the subtype specificity of S-100 assays, about which subtype of S-100 is associated with different clinical entities, and how the results obtained using various assays compare. To answer these questions, we used the Sangtec 100® LIA and the immunofluorometric (IFMA) S-100 assay to analyze purified recombinant monomeric S-100 proteins, purified dimeric S-100 proteins isolated from bovine brain, and blood samples from patients with various CNS diseases, malignant melanoma, or post cardiac surgery and compared the results.

Serum (Sangtec 100 LIA) or heparinized plasma (IFMA S-100) samples were drawn from 218 patients (141 males, 77 females; ages, 16–89 years; mean, 55.4 ± 16.2 years) and 121 healthy blood donors (64 males, 57 females; ages, 18–65 years; mean, 37.8 ± 12.7 years). The study was approved by the local Research Ethics Committee, and all subjects gave informed consent to the procedure. One hundred four of the patients suffered from CNS disease: subarachnoid hemorrhage (n = 41), intracerebral hemorrhage (n = 17), head trauma (n = 19), ischemic cerebral infarction (n = 4), cerebral tumor (n = 12), hydrocephalus (n = 1), or lumbar disc herniation (n = 10). Twenty-nine of the patients had malignant melanoma, and 85 had undergone cardiac surgery involving the use of cardio-pulmonary bypass. Samples were centrifuged within 6 h and stored at −70 °C until analysis. Measurements were performed in duplicate.

The Sangtec 100 LIA immunoluminometric assay uses tubes, coated with two monoclonal antibodies, as solid phase and a monoclonal antibody for detection. The assay measures concentrations of S-100 protein over the range 0–20 μg/L. Measurements were performed according to

References

the instructions of the manufacturer. The IFMA S-100 is an immunofluorometric assay that uses microtiter plates coated with a monoclonal anti-S-100B antibody and a polyclonal detection antibody as described elsewhere (1). A 1:1 (by volume) mixture of S-100A1-B and S-100B-B (Sigma) was used for calibration. S-100 protein concentrations between 0.015 and 25 μg/L were measured.

To determine the assay specificities for different S-100 subtypes, solutions with known concentrations of purified recombinant monomeric S-100A1, S-100B, and the dimeric proteins S-100A1-A1, S-100B-B, and S-100A1-B (Sigma) were measured. S-100A1 and S-100B were produced as described previously (11, 12). Stock solutions contained 6.39 g/L S-100A1, 1.3 g/L S-100B (as determined using the Lowry method); of dimeric S-100A1-A1, S-100A1-B, and S-100B-B as specified by the manufacturer; the measured values in μg/L; and the calculated cross-reactivity in percentage obtained using both assays.

Table 1. S-100 subtype cross-reactivity of the Sangtec 100 LIA and the S-100 IFMA. a

<table>
<thead>
<tr>
<th>S-100 subtype</th>
<th>Actual value, μg/L</th>
<th>Sangtec 100 LIA</th>
<th>S-100 IFMA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Measured value, μg/L</td>
<td>Measured value, %</td>
<td>Measured value, μg/L</td>
</tr>
<tr>
<td>S-100A1</td>
<td>6390</td>
<td>&lt;0.02</td>
<td>&lt;0.015</td>
</tr>
<tr>
<td></td>
<td>63 900</td>
<td>0.029</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>S-100B</td>
<td>0.13</td>
<td>5.04</td>
<td>3877</td>
</tr>
<tr>
<td></td>
<td>1.3</td>
<td>&gt;20</td>
<td></td>
</tr>
<tr>
<td>S-100A1-A1</td>
<td>1000</td>
<td>1.76</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>10 000</td>
<td>12.8</td>
<td>0.13</td>
</tr>
<tr>
<td>S-100A1-B</td>
<td>1.0</td>
<td>13.8</td>
<td>1380</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>&gt;20</td>
<td></td>
</tr>
<tr>
<td>S-100B-B</td>
<td>0.1</td>
<td>2.02</td>
<td>2020</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>4.82</td>
<td>482</td>
</tr>
</tbody>
</table>

* Given are the actual concentrations of monomeric S-100A1 and S-100B as determined using the Lowry method; of dimeric S-100A1-A1, S-100A1-B, and S-100B-B as specified by the manufacturer; the measured values in μg/L; and the calculated cross-reactivity in percentage obtained using both assays.

The results of the S-100 subtype cross-reactivity evaluation are summarized in Table 1. Recovery rates of S-100B protein from analyzed samples were 94.8–113.2% for the Sangtec 100 LIA and 89.7–100.6% for the IFMA S-100. The calculated lower limit for detection for S-100B was <0.015 μg/L in the IFMA S-100 and <0.02 μg/L in the Sangtec 100 LIA. The precision was calculated for concentration ranges of <0.1, 0.1–0.49, 0.5–1.0, and >1.0 μg/L for 222 samples. The median CVs were 15%, 5.7%, 3.7%, and 2.5% for the Sangtec 100 LIA and 7.9%, 3.6%, 3.2%, and 2.2% for the IFMA S-100. The median concentration of S-100 protein in specimens from the blood donors assayed using the Sangtec 100 LIA was 0.014 μg/L (10th percentile, 0.0 μg/L; 90th percentile, 0.089 μg/L). In a previous study using the S-100 IFMA, we found a median plasma concentration of 0.052 μg/L S-100 (10th percentile, 0.023 μg/L; 90th percentile, 0.097 μg/L) in 200 healthy blood donors (ages, 18–65 years) and no dependence of S-100 concentration on age or sex (13). The scatter plot of patient samples and linear regression analyses for each of the three groups and all groups together are shown in Fig. 1. S-100 measurements obtained using the two assays correlated well (R² = 0.95). Comparison of the slopes of the regression lines, however, showed significant differ-

![Fig. 1. S-100 protein was measured in blood samples of 218 patients using both the Sangtec 100 LIA and the IFMA S-100 assay systems. The scatter plot of all samples and the linear regression line are shown. The S-100 measurements obtained using both assays were closely correlated, with R² = 0.95 (intercept, 0.06; slope, 1.59) in all patients; R² = 0.97 (intercept, −0.02; slope, 1.49) in the patients with neurological disease; R² = 0.94 (intercept, 0.07; slope, 1.85) in the patients post cardiac surgery; and R² = 0.99 (intercept, 0.00; slope, 1.89) in the melanoma patients.](image)
ences between S-100 concentrations in the blood of patients with a CNS disorder and patients who had undergone cardiac surgery (t = 6.92; df = 185; P < 0.05, two-tailed) and also between patients with a CNS disorder and patients suffering from malignant melanoma (t = 5.64; df = 129; P < 0.05, two-tailed). The patients who had undergone cardiac surgery did not differ from the melanoma patients (t = 0.60; df = 110; P > 0.05, two-tailed).

To the best of our knowledge, this study is the first to report validation data of the Sangtec 100 LIA assay. Our results showed that both assays are reliable methods with comparable lower limits of detection, measurement ranges, precision profiles, and reference ranges. Both assays are specific for monomeric S-100B. S-100A1 was not detected by both methods. However, the extent of cross-reactivity between the different dimeric proteins (S-100A1-A1, S-100A1-B, and S-100B-B) differed between both methods. Only small amounts of the A1-A1 dimer were detected by both assays, and differences between the methods were small. Although both assays were comparable regarding the measurement of S-100B-B, which has been claimed to be relevant in neurological disorders, there was a clear difference in the measurement of S-100A1-B. The Sangtec 100 LIA returned ~10-fold higher S-100A1-B values than the IFMA S-100, which may be attributable to highly different affinities of the antibodies to the dimeric S-100A1-B molecule. It may be speculated that the conformation of the S-100B molecule is altered during formation of the S-100A1-B dimer. This may increase the affinity of the antibodies used in the Sangtec 100 LIA to their binding sites.

Linear regression analyses of the clinical samples demonstrated a good correlation between both assays. The absolute values of the Sangtec 100 LIA were ~twofold higher than the results of the IFMA S-100, which may be explained by two mechanisms: Because no international reference preparation for S-100 protein is available, this may reflect differences in calibration. In addition, the different subtype cross-reactivities of both methods may contribute to these results.

In immunohistochemical studies using antibodies specific for S-100A1, S-100B, or other S-100 proteins, the distribution of these proteins in human tissues has been reported (10). However, no study has been able to define the extent to which the different S-100 subtypes are released into cerebrospinal fluid and blood in distinct clinical entities. Moreover, for methodological reasons, we do not know whether the S-100 proteins are present in monomeric or dimeric forms in the human body. There are numerous reports in the literature about the measurement of S-100 in different diseases. Several authors have claimed, for example, that CNS damage should increase the concentrations of S-100B-B (1–5, 8, 9, 14, 15), whereas S-100A1-A1 is released from cardiac tissue (16). Controversial results have been reported in malignant melanoma, in which either only S-100B or S-100A1 and S-100B were found to be increased (17, 18). The conclusions of all authors, however, were based only on their usage of polyclonal or monoclonal antibodies against the subunits S-100A1 and S-100B. The subtype specificities of these antibodies for the dimeric S-100 proteins have not yet been studied. To be specific for a dimeric protein (e.g., S-100B-B), an antibody should be able to detect the connection region of the two subunits. There are no reports on such antibodies against S-100 proteins. Thus, it is not clear which forms of S-100 proteins have been found by the investigators cited above.

Using purified recombinant S-100A1 and S-100B proteins, we were able to define the subtype cross-reactivities of two widely used S-100 assays. If the assumption holds true that different S-100 subtypes are present in neurological disease, malignant melanoma, and heart disease, the results of two S-100 assays with different subtype cross-reactivities should differ between these groups. In our study, we were able to show statistically significant differences between the neurologically diseased patients compared with the patients who underwent cardiac surgery or suffered from malignant melanoma. In most clinical studies cited above, the authors have considered the monomeric S-100 subunits to be increased. However, there is evidence that in vivo specific functional properties of S-100B depend on its dimeric status (19). It has been questioned whether the monomeric forms can even exist in solution (6). Therefore, we conclude that although this study utilized assay systems based on monoclonal antibodies directed against S-100B subunits, our data may support the hypothesis that different patterns of dimeric S-100 proteins are present in various clinical entities and thus should be taken into account when interpreting “S-100B” values.

References
12. Engelkamp D, Schäfer BW, Erne P, Heizmann CW. S100os, CAPL, CACY:
Detection of hemoglobinopathies in newborns is critical for the identification of those infants in need of follow-up care [reviewed in Ref. (1)]. For example, infants homozygous for the sickle cell mutation are at greater risk for developing fatal pneumococcal infections and sepsis, which can be prevented by prophylactic antibiotic therapy. At present, the majority of newborn screening for hemoglobin (Hb) variants is done by electrophoresis, isoelectric focusing, or HPLC (2) using Hb extracted from dried blood spots. Nevertheless, detection of adult Hb variants often is complicated by the presence of fetal Hb in neonatal blood. Alternative approaches to these protein-based methodologies have been developed that directly detect the presence of hemoglobinopathy-associated mutations in newborn DNA (3), and some have been adapted to use blood spots (4, 5). Using the S and E mutations in the β-globin gene as examples, we have developed a multiplexed, high-throughput methodology that uses an array of allele-specific fluorescent beads and the Luminex® analyzer (Luminex Corporation, Austin, TX; www.luminexcorp.com). This methodology distinguishes between the S and E alleles and their wild-type counterparts, HbA and non-E, of the β-globin gene in each specimen, making it possible to determine the genotype at each locus.

The methodology is based on the principle that fluorescent microspheres with unique fluorescent profiles, called classifications, can be cross-linked to different analyte-specific reagents and used to create a fluorescence-based array capable of simultaneously assaying multiple analytes in each sample (6). The bead classifications were obtained separately from the Luminex Corporation with surface carboxyl groups for chemical cross-linking to different analyte-specific reagents, which in our studies were 5'-amino-modified oligo-dexynucleotides. As indicated above, each bead classification has a unique spectral address based on its emission ratio when excited by the 635 nm laser in the Luminex® instrument. The Luminex software uses this spectral profile to assign beads to their classifications, and each classification occupies a known position on a dot plot of 658 nm vs 712 nm fluorescence. Thus, multiple bead classifications can be combined in one sample, and the Luminex software processes the fluorescent signals to generate an array of bead classifications on the dot plot of 658 nm/712 nm fluorescence. Determination of the amounts of the different analytes bound to each bead classification is accomplished by coincident excitation of the beads with the 532 nm laser in the Luminex® instrument. Thus, labeling bead-bound analytes with a fluorescent reporter molecule such as phycoerythrin, which emits at 575 nm when excited at 532 nm, produces a third fluorescent signal that allows the amounts of analytes bound to the beads to be quantified. Thus, in each sample, the amounts of multiple analytes can be determined from the emissions of a single fluorescent reporter molecule because the analyte specificity and position of each bead classification in the array is known.

The following oligo-dexynucleotides (ODNs) used in our studies were purchased from Oligos Etc, and the positions of the S and E mutations are indicated in bold: 5'-amino-modified 18mer ODNs for coupling to beads were HbA, GCAGACTTCTCTCCAGGA; HbS, GCAGACTTCTCCACAGGA; non-E, CAGGCGCTACACCAAC; HbE, CAGGCGCTACACCAAC; nonsense (NS), GCACATTGGTCTCGAAGG. 5'-Biotinylated ODNs complementary to HbA, HbS, non-E, HbE, and NS were also used to quality control coupling efficiency of 5'-amino ODNs to the beads.

The five 5'-amino-ODNs were coupled in separate reactions to five different bead classifications. More specifically, 2.5 × 10⁶ beads were coupled to 0.5 nmol of ODN with freshly made 1-ethyl-3-(dimethylaminopropyl)carbodiimide HCl, using the protocol provided by the Luminex Corporation.

PCR was performed after treatment of 1-mm blood-spot punches with methanol and boiling water as described (5). A 327-bp region of the β-globin gene between nucleotides 62010 and 62336 of the GenBank sequence (Accession No. U01317.1) was amplified using Qiagen 10× PCR buffer and Taq polymerase with a 5'-biotinylated PCR primer (ACGGCTGTCATCACTTAG) and an unmodified reverse PCR primer (TCCCAACGTGCCCCATTT). Amplification conditions were 5 min at 95 °C and 35 cycles of 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s, followed by extension for 5 min at 72 °C. PCR amplification was verified by agarose gel electrophoresis using an aliquot of each reaction. Both the S and E loci are present in the