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Rapid immunoassay for the determination of glial fibrillary acidic protein (GFAP) in serum

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Abstract

Background: This study was aimed to develop a sensitive and rapid assay for the determination of glial fibrillary acidic protein (GFAP) in serum and to evaluate the clinical applicability in serum samples from patients with acute stroke.
Methods: The two-site chemiluminometric immunoassay, intended to use in a near-patient setting with a single incubation step (20 min), was used to measure serum samples from healthy blood donors and from patients with brain injury and correlated to serum S100B levels.
Results: The GFAP assay covered a concentration range up to 18 μg/L with an analytical sensitivity of 0.014 μg/L. The intra-assay precision was 3.5% at 1.55 μg/L ($n=20$) and 4.1% at 0.39 μg/L ($n=20$). The inter-assay precision was 3.8% at 9.1 μg/L ($n=10$) and 10.3 % at 0.21 μg/L ($n=9$). Normal controls ($n=46$) showed non-detectable GFAP with a 99% upper limit of <0.04 μg/L. GFAP values were associated with progression and severity of the illness in acute stroke patients.
Conclusions: We have developed an improved assay for the measurement of GFAP levels in serum. Serum GFAP is a potential marker for prognosis and outcome in patients with central nervous system disorders.

Keywords: Glial fibrillary acidic protein; Brain injury; Stroke; Serum; STAT assay

1. Introduction

The determination of brain-specific proteins in cerebrospinal fluid (CSF) or peripheral blood could be helpful to establish brain damage, its severity and the prognosis of the disease.

Upon brain injury, the biochemical markers S100B and neuron-specific enolase (NSE) increase in the CSF as well as in blood and are nowadays studied in several clinical trials [1–4]. These clinical studies showed a significant relation between serum S100B levels and the clinical outcome after stroke [1,2,5,6]. However, S100B is not only expressed in the central nervous system (CNS), but is also expressed by peripheral cell types [4]. The data of various studies in the field of cardiac surgery [7,8] and surgery on multi-traumatized brain injury patients [9,10] as well as patients with bone fractures but no brain injury [11] gave evidence that at least part of the S100B serum concentration might be of extracerebral origin. Therefore, the brain specificity of protein S100B came under debate and as early as 1998 Missler and Wiesmann argued for more specific molecular markers of brain injury [12].

Abbreviations: CSF, cerebrospinal fluid; CNS, central nervous system; CV, coefficient of variance; ELISA, enzyme-linked immunosorbent assay; GFAP, glial fibrillary acidic protein; STAT, short turn around time.

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The finding that glial fibrillary acidic protein (GFAP) is almost exclusively expressed by astrocytes of the CNS [13] makes GFAP a promising candidate for a highly specific brain-damage marker. GFAP is a monomeric intermediate filament protein with a molecular mass between 40 and 53 kDa [13]. The possibility to use GFAP as a brain-specific marker was first demonstrated by the observation that GFAP levels increase in CSF upon acute brain damage [14,15]. Subsequently, it was demonstrated that GFAP can be measured in blood after head injury [16,17]. Furthermore, increased serum GFAP levels were measured upon acute stroke and it was shown that these levels might reflect the underlying pathophysiology of acute cerebral infarcts [18]. All together, these features demonstrate that the measurement of GFAP in serum is a potential tool in the diagnosis and treatment of brain injury.

We here describe the development of an improved immunoassay for the detection of GFAP in serum. This two-site chemiluminometric assay is rapid, sensitive and is highly discriminative between normal controls and patients with head injury. Furthermore, we present data from a clinical trial with acute stroke patients that show a reliable association between short turn around time (STAT)-GFAP values and clinical severity as well as outcome of the disease.

2. Methods

2.1. Materials

For correlation, linearity and stability studies, serum samples from patients with head injury were used. Clinical evaluation of the STAT-GFAP assay is based on a sample of acute stroke patients. To measure GFAP in normal samples, serum was obtained from healthy blood donors who had no previous neurological deficit or other serious disorder.

2.2. GFAP short turn around time (STAT) assay

A flat-bottomed microtiter plate (high binding strip plate, Corning Inc. Corning, NY) was coated with 250 ng/well polyclonal rabbit anti-cow GFAP Ab (total Ig; FD: 10F-2213). After incubation for 18 h at room temperature (RT), the wells were washed four times (MultyWash Advantage, TriContinent Inc, Grass Valley, CA) with 500 μL 10 mM phosphate buffer, pH 7.4, supplemented with 0.25% Tween 20. Subsequently, the wells were blocked with 3% bovine serum albumin and 10% sucrose in phosphate buffer (pH 7.4, 2 h, RT). The blocking agent was removed by inversion and after drying (1 h, 37 °C) the wells were stored at 4 °C.

The assay was performed on the STAT-IntraOperative-platform (Future Diagnostics, Wijchen, The Netherlands) at RT; 100 μL purified bovine spinal cord GFAP antigen (0–18 μg/L; MP Biomedicals Inc., Aurora, OH) or 100 μL serum was added to the coated wells. Subsequently, 150 ng/well polyclonal rabbit anti-cow GFAP tracer Ab (total Ig; FD: 10F-2214) labeled with *N*-(4-Aminobutyl)-*N*-ethyl-isoluminol (Sigma-Aldrich, St. Louis, MO) was added. After 20 min incubation, the microtiter strips were washed four times and were loaded into the microplate luminometer. Per well, 100 μL Activator 1 (luminol-enhancer solution with 1 N NaOH) and 100 μL Activator 2 (contains 0.12% H_2O_2) were injected and relative light units (RLU) were counted for 3 s.

2.3. Method comparison

GFAP levels were measured in serum from patients with head injury using the STAT-GFAP assay and with the GFAP-ELISA described previously [17,18].

2.4. Statistics

For the evaluation of the method agreement the Passing and Bablok regression was used. In addition, correlation coefficients were analyzed by Pearson correlation. For the linearity test we used linear regression analysis (all statistical analysis was performed with Analyse-it Software, Ltd).

3. Results

As shown in Fig. 1, the GFAP assay covered a concentration range between 0 and 18 μg/L. The analytical sensitivity, defined as the intercept of two standard deviations from zero binding of the standard curve, was 0.014 μg/L. Furthermore, the high-dose hook effect was not detected up to 1000 μg/L.

3.1. Precision tests

The within-assay coefficient of variance (CV) was 3.5% at a concentration of 1.55 μg/L ($n=20$) and 4.1% at a concentration of 0.39 μg/L ($n=20$). The between-assay CV

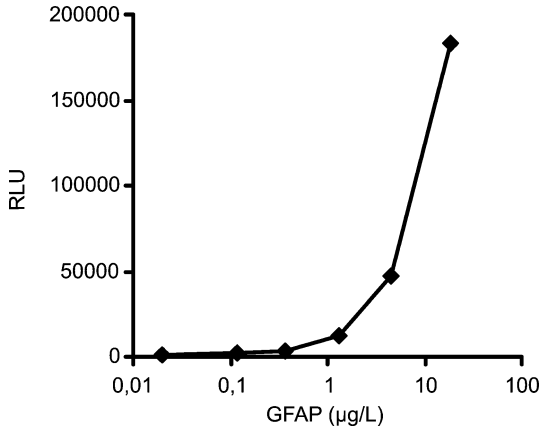

Fig. 1. A typical calibration curve of GFAP. Serial dilutions from 0–18 μg/L. RLU = relative light units.

was 3.8% at a concentration of 9.1 μg/L ($n=10$) and 10.3% at a concentration of 0.21 μg/L ($n=9$).

3.2. Linearity tests

Linearity of the assay was examined by serial dilutions of serum samples from patients with traumatic head injury (S1: 4.6 μg/L, S2: 3.7 μg/L, and S3: 3.3 μg/L), the samples were diluted with a pool of negative serum samples (Fig. 2). The r values were 0.996 ($p=0.0021$), 0.997 ($p=0.0015$), and 0.994 ($p=0.0030$), respectively. Linearity was within 10% of the theoretical sample value.

3.3. Normal controls

Serum samples from 46 healthy donors showed non-detectable GFAP with a 99% upper limit of <0.04 μg/L (Fig. 3).

3.4. Sample stability test

Since GFAP is commonly measured in serum stored at <−20 °C, the effect of freezing and thawing was examined on GFAP levels in 7 serum samples from patients with traumatic head injury (0.14–4.85 μg/L GFAP). A recovery of 100±6% (mean±SD) was obtained. Three samples (S1: 1.2 μg/L, S2: 2.5 μg/L, and S3: 2.9 μg/L) were frozen and thawed for 4 times. GFAP levels were measured after each freeze and thaw cycle. The recovery of S1=97.2±5%, S2=98.9±3% and S3=95.5±1.8%.

3.5. Correlation with GFAP-ELISA

GFAP levels were measured in serum from patients ($n=38$) with head injury. Results obtained with the STAT-GFAP assay were compared with that obtained with the GFAP-ELISA. Passing and Bablok analysis on a retrospective sample correlation revealed a slope of 0.66 in samples with a range up to 9.0 μg/L. The intercept was not significantly different from zero. The Pearson coefficient of correlation was 0.886 ($p<0.0001$).

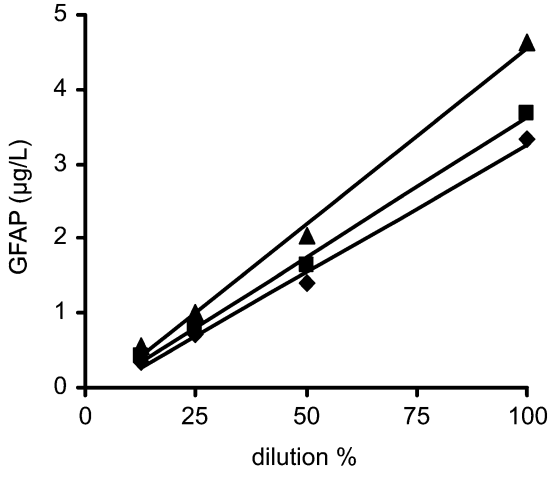

Fig. 2. Dilution curves of three human serum samples from patients with head injury; ▲: 4.6 μg/L, $r=0.996$; ■: 3.7 μg/L, $r=0.997$; and ♦: 3.3 μg/L, $r=0.994$.

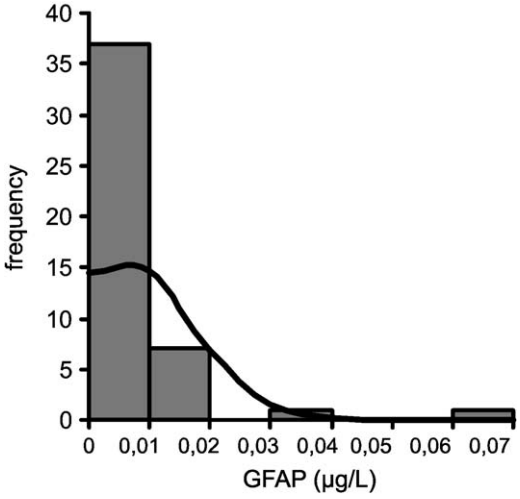

Fig. 3. Frequency of GFAP concentrations in serum of normal controls ($n=46$).

3.6. Clinical evaluation of the STAT-GFAP assay in acute stroke patients

We used serum samples of 22 patients with acute first-ever ischemic stroke in the middle cerebral artery of vascular supply that were obtained shortly after symptom onset (day 1, mean 9 h), and at day 2 (23 h), day 3 (46 h), day 4 (60 h) and day 5 (95 h) at the hospital. Table 1 shows

Table 1
Demographic, clinical and neuroradiological data of stroke patients

Patient characteristics	Clinical parameters
No. of patients	22
Age (years, mean (SD))	67.3 (±10.5)
Sex (male/female, *N* (%))	16 (72.7) / 6 (27.3)
Time between symptom onset and first blood sampling (hours, mean (SD))	9.2 (±6.2)
NIHSS on admission (score, mean (SD))	8.2 (±7.8)
Barthel-score on discharge (score, mean (SD))	71.8 (±37.1)
Lesion volume (cubic centimeters, mean (SD)	32.2 (±81.1)
Stroke subtypes (N (%))[a]	
LACI	4 (18.2)
TACI	8 (36.4)
PACI	5 (22.7)
Not classifiable	5 (22.7)
Etiology (N (%))[b]	
Cardioembolism	5 (22.7)
Small-artery occlusion	6 (27.3)
Large-artery atherosclerosis	5 (22.7)
Undetermined	6 (27.3)

NIHSS = National Institutes of Health Stroke Scale, LACI = lacunar infarcts, TACI = total anterior circulation infarcts, PACI = partial anterior circulation infarcts.

[a] According to Bamford et al. [19].

[b] According to the TOAST-criteria [20].

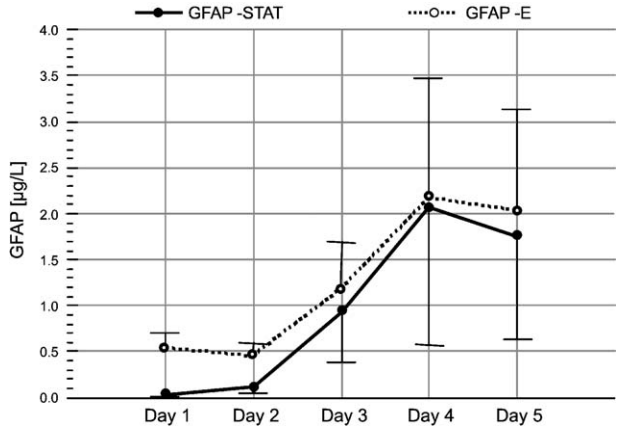

Fig. 4. Release patterns of GFAP in 22 acute stroke patients analyzed with a GFAP-ELISA assay (dashed line, open circles) and the newly developed GFAP-STAT assay (solid line, filled circles). Error bars indicate ±1 SEM.

demographic, clinical and neuroradiological data of the patients' sample used for the clinical evaluation of the STAT-GFAP assay. For method comparisons we analyzed GFAP with both the here described STAT-GFAP assay and the GFAP-ELISA [17] as well as protein S100B. Release patterns of GFAP in all patients is given in Fig. 4. This graph demonstrates that at lower concentrations the functional sensitivity of the STAT-GFAP assay is better than that of the ELISA and that both assays measure the same levels of GFAP at higher concentrations. STAT-GFAP and GFAP-ELISA values were found to be highly and significantly correlated reaching peak correlation coefficients at day 5 ($r=0.99$, $p<0.0001$; all correlation coefficients refer to Pearson correlations, all significance levels are two-tailed). We also found a high and significant correlation between STAT-GFAP and S100B values (day 1: $r=0.97$; day 2: $r=0.77$; day 3: $r=0.88$; day 4: $r=0.90$; day 5: $r=0.97$; all $p<0.0001$). STAT-GFAP values correlated significantly with volume of cerebral lesion (day 5: $r=0.99$; $p<0.0001$) and both severity of neurological disorder (National Institutes of Health stroke scale: day 5: $r=0.79$; $p<0.0001$) and functional outcome (Barthel score: day 5: $r=0.55$; $p=0.009$) at discharge from the hospital. Fig. 5 shows the serum values of STAT-GFAP, GFAP-ELISA and S100B in a patient with progressive stroke who presented with a weak but continuously deteriorating neurological condition. Serial CT examinations at admission to the hospital, and day 4 and day 7 after onset of stroke symptoms, show an increasing area of brain infarction. This figure demonstrates that an increase of GFAP serum concentrations do reflect both clinical deterioration and size of brain lesion.

4. Discussion

In most previous studies that investigated molecular markers of brain disorders in clinical trials, S100B was used as a surrogate marker of brain damage after acute CNS injury such as stroke [1,2,18] or traumatic brain injury [3,21]. However, S100B is not exclusively expressed by astrocytes but also by several peripheral cell types [4] and the analysis of S100B in clinical investigations with diseases not exclusively restricted to the brain was controversially discussed. As a consequence, GFAP might be a more specific and promising marker for diagnosis as well as for monitoring the efficacy of neuroprotective treatment in patients with CNS and concomitant extracerebral disorders.

Here, we describe a sensitive chemiluminescent STAT assay for the measurement of GFAP levels in serum. Compared to the GFAP-ELISA that was developed previously [17], the current STAT-GFAP assay has a 10-fold higher analytical sensitivity (0.014 µg/L). In addition, the STAT-GFAP assay showed virtually non-detectable GFAP in serum of healthy blood donors (<0.014–0.066 µg/L), whereas Van Geel et al. found <0.15–0.76 µg/L GFAP in serum from normal controls [17]. Importantly, the values of positive samples were highly correlated between the two methods. In contrast, Missler et al. reported a very sensitive assay but also relatively low signals for positive serum samples from patients with head injury [16]. They found increased GFAP in 12 out of 25 serum samples from patients with head injury, with approximately 10-fold lower expression levels as S100B. In addition, GFAP could be detected 24 h after injury in only 1 out of 25 serum samples,

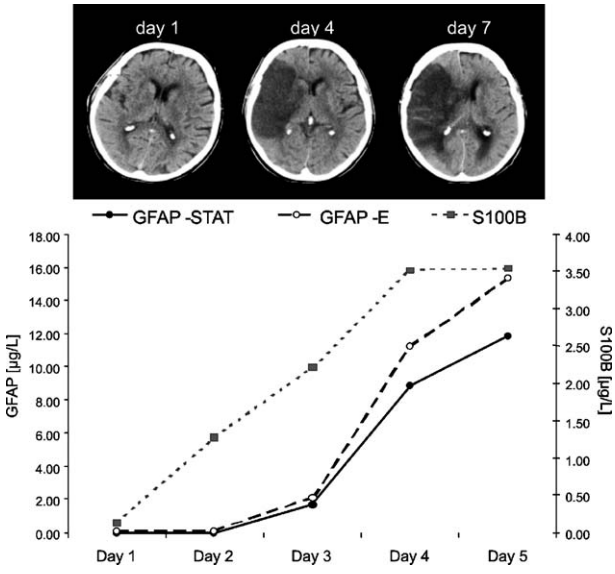

Fig. 5. Illustration of GFAP and S100B values in one patient with a progressive stroke comprising the whole middle cerebral artery territory of vascular supply. Upper part: serial CT images obtained from the patient shortly after admission, at day 4 and day 7, after stroke onset. Imaging data show a diffuse right temporal gyral swelling with asymmetric sulcal effacement on day 1 and a total infarction of the right middle cerebral artery territory of vascular supply (day 4) with signs of hemorrhagic transformation (day 7). Lower part: serum values of S100B (dashed line, filled squares), GFAP-ELISA (dashed line, open circles) and STAT-GFAP (solid line, filled circles) obtained from this patient from day 1 to day 5 after onset of stroke symptoms.

while others showed that serum GFAP and S100B levels are comparable and found a GFAP peak at 24 h after head injury [17,21].

The clinical evaluation of the STAT-GFAP assay showed that GFAP concentrations in patients with acute stroke are highly and significantly associated with the volume of brain lesion and both severity of the neurological disorder and functional status at discharge from the hospital. Thus, STAT-GFAP values do reflect the development of the disease and are associated with clinical outcome. The comparative analysis of serial GFAP serum concentrations in a single case as well as in a group of stroke patients demonstrates the highly reliable clinical performance of the STAT-GFAP assay.

Taken together, our data demonstrate that the STAT-GFAP assay is a powerful tool to detect GFAP in serum, even at low levels. Furthermore, the STAT platform makes it possible to determine GFAP in a near-patient situation where the availability for normal laboratory equipment is limited and to determine a small series of patient samples.

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