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## Measurement of S-100 Protein in Human Blood and Cerebrospinal Fluid: Analytical Method and Preliminary Clinical Results

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**Summary:** An immunofluorometric sandwich assay for determination of S-100 protein in cerebrospinal fluid (CSF) and blood is described. The lower detection limit was 0.015 µg/l of S-100 protein. Intra-assay and inter-assay imprecision (coefficients of variation, CVs) were 2.1 to 3.2% and 7.8 to 11.6%, respectively. S-100 protein recovery in cerebrospinal fluid was 94 to 103%. In blood the recovery varied from 67 to 96%, depending on blood samples used and the concentration of S-100 protein. The best recovery in blood was found using heparinized plasma. In healthy subjects  $0.098 \pm 0.11$  µg/l (mean  $\pm$  SD) of S-100 protein was detected ( $n = 30$ ). In the CSF of otherwise healthy patients undergoing a myelography for lumbar pain  $1.43 \pm 0.49$  µg/l (mean  $\pm$  SD) of S-100 protein was found. Preliminary results from longitudinal studies on S-100 protein in neurosurgical patients indicate a positive correlation between S-100 protein blood levels and clinical course. Thus, the determination of S-100 protein in blood appears to be helpful in the monitoring of patients with neuronal damage.

### Introduction

Every physician involved in the care of neurologically diseased patients in intensive care medicine faces the problem of evaluating patients in coma or under general anaesthesia. In many of these cases, classical neurological examination alone does not allow sufficient monitoring on the intensive care unit for therapeutic guidance or allow early diagnosis of complications. Even computed tomography (CT) and nuclear magnetic resonance (NMR) imaging are not always able to differentiate oedema from structurally damaged neuronal tissue. This is especially true in the acute phase of brain damage. In addition, neuroradiological techniques are expensive and require transportation, which is not without risk for critically ill patients. Monitoring of intracranial pressure, electroencephalogram (EEG) or sensory evoked response are valuable tools and can provide important information but do not prove neuronal destruction. In addition, in intensive care units these methods may be susceptible to technical interference. There is evidence, however, that cerebrospinal fluid markers such as S-100 protein, glial fibrillary acidic protein, neuron-specific

enolase and myelin basic protein may serve as quantitative markers of the extent of brain damage.

The term "S-100" refers to a mixture of dimeric proteins consisting of two subunits of  $M_r$  10 500 termed  $\alpha$  and  $\beta$  (1). Three isoforms are known. S-100<sub>a</sub> ( $\alpha\beta$ ) is found in glial cells and melanocytes. S-100<sub>b</sub> ( $\beta\beta$ ) is present in high concentration in glial cells and Schwann cells of the central and peripheral nervous system as well as in Langerhans cells and cells of the anterior pituitary. S-100<sub>ao</sub> ( $\alpha\alpha$ ) which represents 5% of the S-100 protein population in brain is found outside the nervous system in slow-twitch muscle, heart and kidney (2). The S-100 protein family constitutes a subgroup of  $Ca^{2+}$ -binding proteins of the EF-hand type (3). Apart from calcium they can also bind zinc. Both intracellular and extracellular mechanisms of action have been proposed for S-100 protein, although its biological functions are not yet understood in detail (3). S-100 protein is stable in CSF for 2 days at 4 °C (4).

Several studies on patients with neurological lesions have shown a relationship between cell damage in the

central nervous system and the concentration of S-100 protein in cerebrospinal fluid (CSF) (5–13). However, especially in patients with substantial lesions of the brain (large tumour, infarction, intracerebral or subarachnoid haemorrhage, neurotrauma) intracranial pressure may be increased and lumbar puncture is contraindicated due to the risk of transtentorial herniation. CSF can be obtained by cannulation of the lateral ventricles. This is, however, not without risk and during cannulation nervous tissue will be damaged, which may distort measurements (14, 15).

For these reasons, substances which are released from brain cells during brain damage must be detectable in blood if they are to serve as a useful tool in clinical medicine. In contrast to CSF, however, studies on S-100 protein levels in blood are rare (9). A correlation between S-100 protein levels in blood and damage of the nervous system has not previously been reported. We describe a method for quantifying S-100 protein concentrations in blood and CSF and report the first clinical results obtained with the assay under routine conditions.

## Materials and Methods

### Samples

#### Reference population

CSF was obtained from 53 patients (25 male, 28 female) aged 17–76 years ( $55.2 \pm 14.2$ , mean  $\pm$  SD) who underwent myelography for lumbar pain. Medical history and neurological evaluation revealed no evidence for organic nervous disease. Total protein concentration and cell count in CSF specimens were both normal. Serum and heparinized plasma samples (ammonium heparin,  $15 \times 10^3$  IU/l) were obtained from 30 healthy subjects (15 male, 15 female) aged 21–52 years ( $32.5 \pm 7.7$ , mean  $\pm$  SD).

#### Neurosurgical patients

Heparinized plasma and, if available, CSF samples from lumbar punctures, lumbar catheters and lateral ventricle cannulations were obtained for routine analysis from patients of the neurosurgical intensive care unit of the Medical University of Lübeck.

CSF samples from lateral ventricle cannulation of four patients were selected for dilution and analytical recovery experiments. Eleven serum and heparinized plasma samples of healthy subjects were selected for the calcium dependency experiments, and a further 12 samples for dilution studies. All dilutions were made in horse serum (Gibco, Eggenstein, Germany). All blood CSF samples were centrifuged within three hours and the supernatant stored in aliquots of 500  $\mu$ l at  $-70^\circ\text{C}$ .

### Materials

All chemicals were obtained from Merck (Darmstadt, Germany) unless stated otherwise. Purified S-100<sub>a</sub>, S-100<sub>b</sub> and S-100<sub>ab</sub> proteins from Sigma (Deisenhofen, Germany) were used for S-100 protein subtype cross-reactivity experiments and as calibrator.

### Antibodies

The polyclonal antibody was raised in rabbit with bovine brain S-100 protein purified by the method of Moore (1) as antigen

(DAKO, Hamburg, Germany). After plasma adsorption the specificity of the antibody was proven by immunoelectrophoresis and enzyme immunoassay. The antibody was labelled with biotin as described previously (16).

### Assay procedure

Microtitre plates (Nunc Maxisorp, Roskilde, Denmark) were coated with anti-S100 protein antibody (DAKO) 900 ng/well in 200  $\mu$ l phosphate buffer, 0.05 mol/l, pH 8.6 and allowed to stand overnight at  $4^\circ\text{C}$ . After washing the plates twice with 5 ml/well of washing buffer (Tris 0.05 mol/l, NaCl 0.15 mol/l, Tween 20 0.1 ml/l, pH 7.5) using a Novopath Platewasher (Bio Rad, München, Germany) they were postcoated for 15 minutes with 300  $\mu$ l of carbonate buffer 0.05 mol/l, pH 9.6 containing bovine serum albumin 10 g/l (Sigma) and washed again once with washing buffer. The plates were either used fresh, or were stored at  $4^\circ\text{C}$  with 200  $\mu$ l coating buffer per well and sealed with microtitre plate sealings (ICN, Eschwege, Germany). The plates could be stored in this way for 12 weeks without loss in immunoreactivity.

All measurements were set up in duplicate at room temperature. Incubations were performed on a microtitre plate shaker (Heidolph, Kehlheim, Germany) at  $200\text{ min}^{-1}$ . CSF samples were measured undiluted and after 1 : 100 dilution in horse serum. S-100 protein calibrators, controls, samples (200  $\mu$ l) and 50  $\mu$ l of horse serum (Gibco) with  $\text{CaCl}_2$  25 mmol/l were added to each well and incubated for 2 hours. Purified S-100<sub>a/b</sub> (1 + 1, by vol.) in horse serum was used for calibrators and controls. The plates were washed with washing buffer three times, and 200  $\mu$ l of biotin-labelled anti S-100 protein antibody diluted to a final concentration of 2  $\mu$ g/l in a Tris 0.05 mol/l, NaCl 0.15 mol/l,  $\text{CaCl}_2$  10 mmol/l,  $\text{NaN}_3$  0.15 mmol/l buffer containing horse serum, volume fraction 0.5, was added and incubated for one hour.

The plates were again washed three times with washing buffer, after which 200  $\mu$ l of streptavidin-europium were added to each well and incubated for 30 minutes. Streptavidin was labelled as recommended by Pharmacia (Uppsala, Sweden) using their europium-labelling-reagent, and then diluted to a final concentration of 0.025 mg/l in Tris 0.05 mol/l, NaCl 0.15 mol/l,  $\text{NaN}_3$  0.15 mmol/l, diethylenetriaminopentaacetic acid 0.02 mmol/l, bovine serum albumin 5 g/l and bovine  $\gamma$ -globulin 0.5 g/l (Sigma), pH 7.5. The plates were washed again three times with washing buffer. Enhancement solution (200  $\mu$ l/well; acetic acid 0.01 mol/l, tri-n-octyl phosphine oxide 38 mg/l, potassium phthalate 1.3 g/l, thenoyltrifluoroacetone 222 mg/l, Triton X-100 2 mol/l) was incubated for 15 minutes. The resulting fluorescence was measured in the DELFIA 1232 fluorometer (Pharmacia). The calibration curve was constructed using a modified spline function in the FIA-Calc data reduction program of the DELFIA 1232 system after a log/log transformation of the data.

### Effect of calcium concentration on the S-100 protein assay

To evaluate the effect of calcium concentration on the S-100 protein assay we obtained serum and heparinized plasma samples from 11 healthy subjects. S-100<sub>a/b</sub> (1 + 1, by vol.) and  $\text{CaCl}_2$  was added to give final concentrations of 1 and 10  $\mu$ g/l of S-100 protein and 2, 5, 10 and 20 mmol/l of  $\text{CaCl}_2$ , respectively. For this experiment the buffer solutions described above did not contain calcium.

## Results

### Assay characteristics

A typical calibration curve of the S-100 protein assay is shown in figure 1. The lower detection limit was 0.015

$\mu\text{g/l}$  ( $0 + 3$  SD,  $n = 24$ ) of S-100 protein. The calibration curve ranged from 0.02 to 25  $\mu\text{g/l}$ . A high dose "hook"-effect did not occur even at S-100 protein concentrations of 10 000  $\mu\text{g/l}$  (fluorescence reading for calibrator 25  $\mu\text{g/l}$ : 1 419 080  $\text{s}^{-1}$ , 10 000  $\mu\text{g/l}$ : 2 483 335  $\text{s}^{-1}$ ). Coated microtitre plates stored in buffer for 3 months at 4 °C gave the same results as freshly prepared plates. The lower detection limit of the assay depended on the calcium concentration (tab. 1). With increasing  $\text{CaCl}_2$  concentrations up to 10 mmol/l, the measured S-100 levels in serum and heparinized plasma of healthy volunteers also steadily increased. Since the best analytical recovery after addition of S-100 protein to blood was achieved with 5 mmol/l of  $\text{CaCl}_2$ , this calcium concentration was chosen for assay buffers. S-100 protein in heparinized plasma was stable for 24 hours at 20 °C (10% loss), for four days at 4 °C (without loss) and for at least 6 months at -20 °C. Even four cycles of freezing and thawing did not affect S-100 levels in plasma (data not shown).

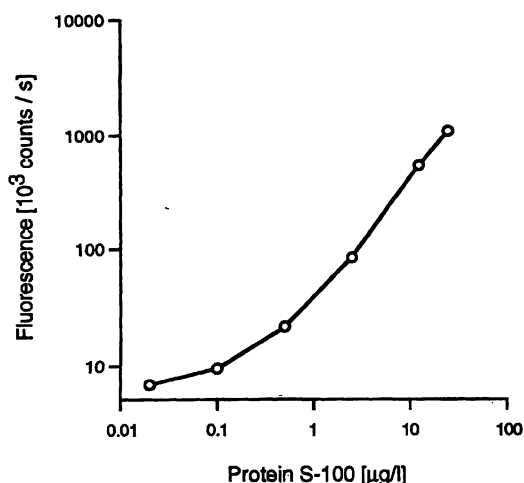


Fig. 1 Typical calibration curve of the S-100 protein assay.

Tab. 1 S-100 protein concentrations in serum and heparinized plasma of 11 healthy subjects after addition of calcium and S-100 protein.

Calcium added [mmol/l]	S-100 protein added [ $\mu\text{g/l}$ ]		
	0	1	10
<b>Serum (n = 11)</b>			
—	<0.02	0.40	4.44
2	<0.02	0.51	5.11
5	0.03	0.62	6.30
10	0.046	0.65	6.02
<b>Heparinized plasma (n = 11)</b>			
—	0.063	0.76	7.97
2	0.068	0.75	7.76
5	0.083	0.75	8.48
10	0.092	0.66	7.30

## Precision

The intra-assay (within-run) imprecision (CVs) was 3.2% at 0.51  $\mu\text{g/l}$ , 2.1% at 5.97  $\mu\text{g/l}$ , and 2.3% at 11.4  $\mu\text{g/l}$  ( $n = 20$ ). The total imprecision (between-day, CVs) was 11.5% at 0.45  $\mu\text{g/l}$ , 7.9% at 4.79  $\mu\text{g/l}$ , and 7.8% at 15.45  $\mu\text{g/l}$  ( $n = 21$ ).

## S-100 subtype cross reactivity

The assay detected predominantly S-100<sub>b</sub>. Cross reaction by S-100<sub>ao</sub> was less than 1% (tab. 2).

## Recovery experiments

The recovery after mixing different CSF samples varied from 94% to 103% (tab. 3). Serial dilution experiments proved the linearity of the assay (figs. 2 and 3). The results were different for serum and heparinized plasma (tab. 4).

## S-100 protein reference values

The CSF samples of the reference group contained 1.43  $\pm$  0.49  $\mu\text{g/l}$  of S-100 protein (mean  $\pm$  SD). Values exceeding 2.4  $\mu\text{g/l}$  (mean + 2 SD) were considered elevated. In the serum of healthy subjects 0.026  $\pm$  0.057

Tab. 2 100  $\mu\text{g/l}$  of S-100<sub>ao</sub>, 25  $\mu\text{g/l}$  of S-100<sub>a</sub> and 12.5  $\mu\text{g/l}$  of S-100<sub>b</sub> diluted in horse serum were measured in the S-100 protein assay using S-100<sub>a</sub>: S-100<sub>b</sub> as 1 + 1 (by vol.) as calibrator. The percentage of cross reactivity was calculated.

S-100 protein subtype	Concentration		Cross-reactivity [%]
	expected [ $\mu\text{g/l}$ ]	detected [ $\mu\text{g/l}$ ]	
S-100 <sub>ao</sub>	100	0.89	0.90
S-100 <sub>a</sub>	25	2.91	12
S-100 <sub>b</sub>	12.5	21.3	170

Tab. 3 Four different CSF samples were mixed, measured in the S-100 protein assay and the analytical recovery was calculated as a percentage. The S-100 protein concentration of the samples was 20.0  $\mu\text{g/l}$  for specimen 1, 0.62  $\mu\text{g/l}$  for specimen 2, 4.00  $\mu\text{g/l}$  for specimen 3 and 8.20  $\mu\text{g/l}$  for specimen 4.

Combination of specimens	Concentration		Recovery [%]
	expected [ $\mu\text{g/l}$ ]	detected [ $\mu\text{g/l}$ ]	
1 + 2	10.3	10.1	98
1 + 3	12.0	12.0	100
1 + 4	14.1	13.3	94
2 + 3	2.31	2.25	97
2 + 4	4.41	4.40	100
3 + 4	6.10	6.30	103

$\mu\text{g/l}$  of S-100 protein was found (mean  $\pm$  SD). Concentrations in heparinized plasma ( $0.098 \pm 0.11 \mu\text{g/l}$ , mean  $\pm$  SD) were nearly four times as high as in serum. Only 6 of the 30 serum reference samples were within the

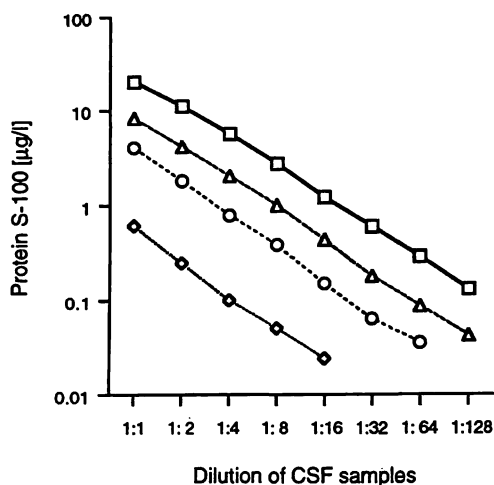


Fig. 2 Serial dilution of four CSF samples in horse serum.

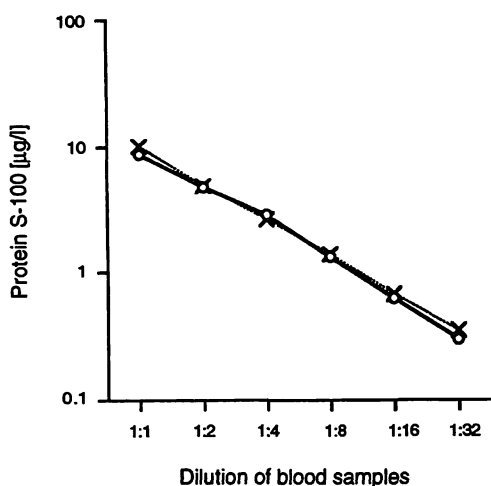


Fig. 3 S-100 protein was added to serum and heparinized plasma of 12 healthy subjects to give a concentration of  $10 \mu\text{g/l}$ . Shown are the mean values of measured S-100 protein for serum (O) and heparinized plasma (x) after serial dilution in horse serum.

Tab. 4 Measured concentrations of S-100 protein after addition of 1 and  $10 \mu\text{g}$  of S-100 protein to serum and heparinized plasma of 30 healthy subjects. In both incubation steps calcium was added to give a concentration of  $5 \text{ mmol/l}$ .

	Recovery after addition of S-100 protein	
	1 $\mu\text{g/l}$ added	10 $\mu\text{g/l}$ added
Serum (n = 30)		
Mean $\pm$ SD	$0.669 \pm 0.47$	$7.20 \pm 1.43$
Heparinized plasma (n = 30)		
Mean $\pm$ SD	$0.775 \pm 0.142$	$9.56 \pm 1.58$

calibration range. In contrast, 22 of the 30 heparinized plasma samples were within the calibration range.

S-100 protein concentrations in blood and CSF were measured during the clinical course in neurosurgical patients. To illustrate the possible diagnostic value of the method described here one individual course of a patient suffering from subarachnoid haemorrhage is presented in figure 4.

## Discussion

Several groups have reported increased cerebrospinal fluid levels of S-100 protein in patients with lesions of the central nervous system (7–13). There is evidence that CSF levels of S-100 protein and other proteins (glial fibrillary acidic protein, neuron-specific enolase) may serve as quantitative markers of the extent of brain damage (12, 15, 17–20).

In 1987 Persson et al. (9) reported the determination of S-100 protein in CSF and serum, using a radioimmunoassay with rabbit and bovine S-100 protein antibody from DAKO. In the serum of 16 healthy controls they found S-100 protein levels of  $\leq 5.3 \mu\text{g/l}$ . In the clinical course of one patient with cerebral infarction they also determined three serum S-100 protein levels within the first 48 hours after onset of symptoms. In this particular case, an increase of S-100 protein levels in blood was demonstrated for the first time. Other reports on S-100

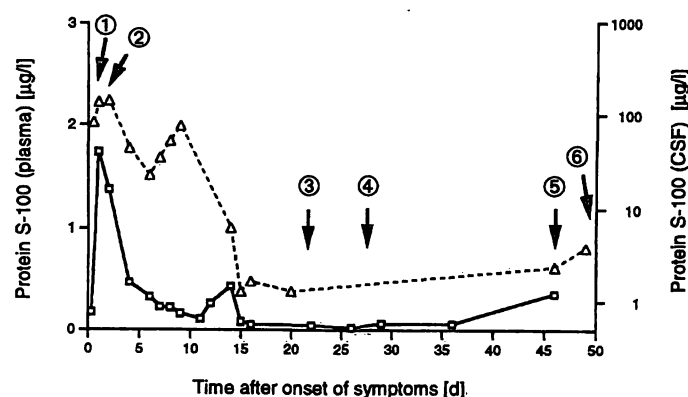


Fig. 4 Plasma (□) and CSF (Δ) S-100 protein concentration course from a 42-year old female with a severe subarachnoid haemorrhage with hydrocephalus from a ruptured aneurysm of the anterior communicating artery. Admitted in coma with signs of a tegmental syndrome.

- ① Ventricular cannulation.
- ② Clipping of the aneurysm.
- ③ Due to prolonged brain oedema general anaesthesia with barbiturate (methohexital) was performed until day 21 after the operation.
- ④ Patient breathed spontaneously. The neurological state improved slowly.
- ⑤ Bacterial meningitis.
- ⑥ Transfer to a rehabilitation unit. At this time she was able to communicate, still suffering from a psychosyndrome.

protein blood levels in patients with brain damage could not be found in the literature.

The method described measures S-100 protein levels in blood and CSF with adequate sensitivity and reliability. The reference range found for CSF ( $\leq 2.4 \mu\text{g/l}$ ) corresponds well with the values reported in the literature (7, 13, 21). Several factors may contribute to the clear differences between the reference values for blood reported by *Persson et al.* (9) and those reported in the present study. *Persson* did not describe the calibrator he used. The lower detection limit of their method is  $1 \mu\text{g/l}$ , compared with  $0.02 \mu\text{g/l}$  in our assay. Therefore, the main reason may be differences in the calibration. As long as no international standardisation of S-100 protein quantification exists, the comparability of absolute values must remain questionable.

For ethical reasons it is not possible to obtain CSF from healthy volunteers to establish a reference range for normal individuals. Instead we selected patients undergoing myelography for lumbar pain. Neurological examination and medical history revealed no evidence of an organic disease of the central nervous system. Nevertheless this selection remains a compromise. For ethical reasons we also did not obtain ventricular and lumbar CSF simultaneously. Thus we cannot give data concerning the comparability of the two sources. However, *Aurell et al.* (4) obtained 50 ml of lumbar CSF from patients with normal pressure hydrocephalus in consecutive portions of 10 ml and found no differences in the S-100 protein levels between the five portions.

The effect of calcium on the measurement of S-100 protein has already been described by *Sindic et al.* (13). We found the same optimal calcium concentration as *Sindic et al.*, and the addition of  $5 \text{ mmol/l CaCl}_2$  decreased the lower detection limit of our assay. Possible explanations of the incomplete recovery of S-100 protein from blood are S-100 protein binding proteins, S-100 protein digesting enzymes or poor affinity of the antibody used. The latter, however, appears unlikely since horse serum was used as diluent for calibrators, controls and all dilutions. Horse serum was also used to dilute CSF, in order to minimize matrix effects (22). Horse serum was used in preference to newborn calf serum, because newborn calf serum contains high levels of S-100 protein which react with the antiserum, particularly since this was raised against bovine S-100 protein. To calculate the analytical recovery from CSF a classical mixing experiment was performed. The problem of measuring S-100 protein in blood is the poor recovery rate, which may be the reason why reports on S-100 protein measurements in blood are so rare in the literature. Therefore, we also performed a recovery experiment in which fixed amounts of S-100

protein were added to the serum and heparinized plasma of 30 normal individuals.

Whether decreasing calcium levels during blood coagulation are the only reason for the different recoveries in serum and plasma remains unclear. It is, however, well known from the literature that calcium induces a conformational change in the S-100 protein molecule and thus may affect antibody binding (23). In spite of the fact that calcium is clearly more effective in improving the recovery of S-100 protein from serum, the absolute recovery rates from heparinized plasma remain higher. This may be caused by unknown factors in serum.

Recovery experiments in EDTA and citrate plasma failed, because the addition of calcium induced coagulation. The recovery observed in the experiments to optimize calcium concentration was lower than that obtained later when  $5 \text{ mmol/l CaCl}_2$  was added to all assay buffers.

To assess the clinical importance of the different S-100 protein subtypes (a, b, ao) specific assays must be developed. Our assay predominantly detected S-100<sub>b</sub>. With reference to the distribution of S-100 protein subtypes described above, this assay appears to be suitable for studying central nervous system disease.

These preliminary results of S-100 protein measurements in neurosurgical patients may indicate a positive correlation between S-100 protein plasma levels and clinical course. In the clinical data shown in figure 4 the course of S-100 protein levels in blood and CSF appears to be similar; the slight differences in blood and CSF may be due to the time-delay caused by the blood-brain barrier.

In patients with severe polytrauma, S-100<sub>ao</sub> from outside the CNS can influence the results of S-100 protein measurements (24, 25). Despite the fact that S-100<sub>ao</sub> is only detected to less than 1% with our method, an influence on the results obtained with this assay cannot be completely excluded, although it is unlikely to influence the clinical value of the results.

The assay described above allows a rapid, reliable and non radioisotopic measurement of S-100 protein in blood and CSF. Determination of S-100 protein levels in blood may serve as a useful tool for screening and monitoring patients suffering from lesions of the central nervous system. Assessment of the clinical value of this method will require prospective studies with large patient populations.

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