

A new nephelometric assay for β -trace protein (prostaglandin D synthase) as an indicator of liquorrhoea

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Abstract

Objectives—To determine the sensitivity and specificity of a nephelometric β -trace protein assay for the diagnosis of liquorrhoea.

Methods—One hundred and forty clinical samples with suspected liquorrhoea were analysed by a newly developed nephelometric assay. An established electroimmunoassay served as a reference method. The sensitivity and specificity of the β -trace nephelometric assay were calculated by a 2x2 contingency table for 10 different versions of a dichotomised nephelometric variable. In 52 patients (79 samples), the nephelometric findings were validated by referring to the clinical diagnosis based on the course of the disease, imaging techniques, and surgical inspection.

Results—Given a specificity of 100%, a β -trace protein concentration of 6 mg/l or higher in a sample indicated liquorrhoea with a sensitivity of 92% compared with the reference method and of 93% compared with the clinical evaluation. The relation between the electroimmunoassay and the nephelometric assay was highly significant ($p < 0.001$).

Conclusions—The nephelometric β -trace protein assay is a simple and rapid method for the detection of liquorrhoea with high sensitivity and specificity and may facilitate the diagnosis of fistulas leaking CSF.

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Keywords: β -trace protein; fistulas leaking CSF; liquorrhoea

The early diagnosis of fistulas leaking CSF is of great importance as they may lead to bacterial meningitis, which is a life threatening disease with a mortality rate of 25%–50%. About 20% of cases of bacterial meningitis are due to a CSF leak.¹ Fistulas leaking CSF are sequelae of skull fractures or surgical procedures at the anterior or middle cranial fossa, the nasal sinus, or the temporal bone. Their frequency after temporal bone fracture was found to be 15%.² The aetiology of liquorrhoea remains obscure in up to 39% of cases, including those with spontaneous liquorrhoea.³ Rare conditions leading to fistulas leaking CSF are intracranial hypertension, sphenoidal sinusitis, bromocriptine therapy of macroprolactinoma, and malformations of the inner ear.⁴⁻⁷ Such fistulas

should be suspected in patients presenting with intermittent or permanent water-like secretion of the nose or—to a lesser extent—of the ear, which may be confounded with rhinitis or otitis. In some patients, orthostatic headache may be present as well. The first aim of the diagnostic procedures is to prove the presence of CSF in a given sample. The second step is the location of the fistula using either high resolution CT, CT cisternography, MRI, endoscopy, or detection of radioactive tracer or fluorescein dye previously injected into the lumbar subarachnoidal space.⁸ In a third step the dural defect has to be closed by surgery.

Felgenhauer *et al* demonstrated that an ideal marker for liquorrhoea should be present in normal CSF but not in other body fluids and should be detectable rapidly with routine laboratory methods.⁹ β -Trace protein was introduced by Felgenhauer *et al* as a marker for liquorrhoea because its concentration in normal CSF is 35-fold higher than in serum. The protein is absent in tear fluid or nasal secretion. This protein belongs to the lipocalin family, a group of carrier proteins with enzymatic function. After determination of its amino acid sequence, it became evident that β -trace protein is identical with prostaglandin D-synthase.¹⁰ The physiological role of β -trace protein, which is produced in the meninges, the choroid plexus, and to a lesser extent in astrocytes, is not known.¹¹⁻¹³ Animal experiments point to a role in sleep regulation and nociception.^{14,15} β -Trace protein in CSF has been studied in various CNS diseases but is of limited value except for the differential diagnosis of blood-brain barrier disturbances.¹⁶⁻²¹ By contrast, β -trace protein electroimmunoassay is an important tool with a sensitivity of 97% and specificity of 100% in the diagnosis of fistulas leaking CSF.^{9,22} Here we present a study of 140 liquid samples suspected of containing CSF which were investigated for β -trace protein using a new nephelometric assay.

Methods

SAMPLES

Samples (140) from 100 patients from neurological (9%), neurosurgical (19%), otolaryngological (57%), and other (15%) departments with clinically suspected liquorrhoea were collected during 2 years and investigated with electroimmunoassay and nephelometric assay. Sampling of ear or nose secretion, ear or nose tamponades, intraoperative secretion suspected of containing CSF, or postoperative

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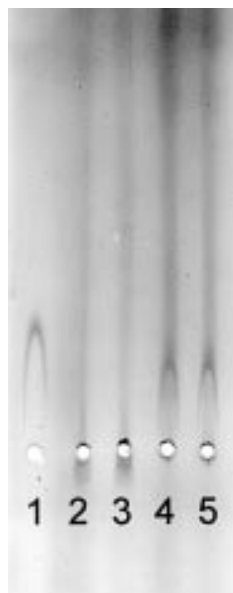


Figure 1
Electroimmunoassay for β -trace protein. 1: positive control (CSF); 2 and 3: negative control (serum); 4 and 5: positive samples (nasal secretion).

hygroma was performed. Sensitivity and specificity of the nephelometric assay were calculated referring to the electroimmunoassay results.

Additionally, the clinical records of 79 of the 140 samples were reviewed for radiological or surgical findings indicating fistulas and compared with the results of the β -trace protein assay. These 79 samples were from 52 patients, 32 men and 20 women, with a mean age of 48 years and a range between 8 and 85 years. All patients had a serum creatinine within the normal range.

Twenty CSF samples of patients presenting with tension headache who showed a normal CSF analysis were analysed for β -trace protein, as well as serum samples from 34 normal volunteers. The mean age (SD) of the 20 patients was 45 (15.8) years. The mean cell count was 1 (SD 0.9)/ μ l and the mean serum/CSF albumin ratio was 223.9 (SD 84.3).

β -TRACE PROTEIN ELECTROIMMUNOASSAY

Anti-human β -trace protein antibody production was induced in rabbits⁹ and detection of β -trace protein was performed by electroimmunoassay as described previously.²³ Briefly, Gelbond plates (Sigma, Munich, Germany) were coated with gel containing 1.5 % Litex agarose (Litex, Denmark), 1% polyethylene glycol 6000 (Merck, Darmstadt, Germany), 0.02 M barbital buffer pH 8.6, and 1% antibody; 5 μ l of sample and a positive and negative control were placed in preformed holes. Samples of CSF served as positive controls, serum samples as negative controls. Electrophoresis was performed with a 0.02 M barbital buffer pH 8.6 for 3 hours at 4°C and 250 V. Complexes of β -trace protein and its antibodies were detected by 0.5% Coomassie blue (Serva Heidelberg, Germany). The detection limit of the method is 5 mg/l. A sample was judged as positive if β -trace protein-protein antibody complexes were present in the typical rocket formation (fig 1).

β -TRACE PROTEIN NEPHELOMETRIC ASSAY

For determination of β -trace protein a newly developed nephelometric research assay (N latex β TP) was used. N Latex β TP (Dade Behring, Marburg Germany) is a lyophilised reagent for Behring nephelometer (BN) systems. It contains polystyrene particles coated with immunoaffinity purified polyclonal antibodies from rabbits against human β -trace protein, which are agglutinated in the presence of β -trace protein. The increase in light scattering caused by agglutination is measured by the BN.

Samples (5 μ l) are diluted to a total volume of 500 μ l with diluents buffer (Dade Behring, Marburg, Germany) and measured on a nephelometer. Twelve minutes after addition of N latex β TP light scattering is measured again. The reaction kit consists of 50 μ l polystyrene particles coated with 1.8 mg/100 mg immunoaffinity purified polyclonal rabbit anti-human β -trace protein antibodies, 15 μ l

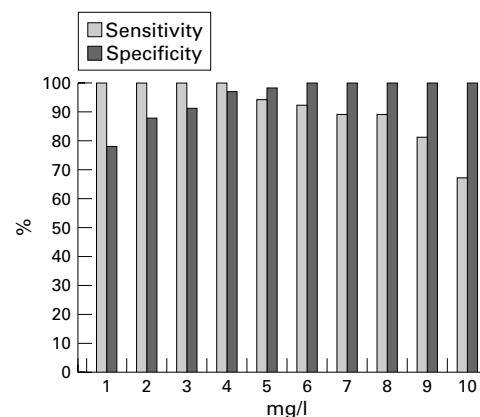


Figure 2 Sensitivity and specificity of β -trace protein nephelometric assay in comparison with the electroimmunoassay for 10 critical concentrations of β -trace protein.

supplement to avoid interference with anti-IgG-antibodies (Dade Behring, Marburg, Germany), and diluents buffer. The concentration of β -trace protein is calculated by the BN software using a seven point standard curve prepared automatically from a single calibrator containing native human β -trace protein. Standardisation of the N Latex β TP assay is based on highly purified β -trace protein from CSF characterised by amino acid sequencing and quantified by quantitative amino assay analysis. The measuring range is 0.25 to 15.8 mg/l for the original dilution of 1:100. Samples with higher or lower β -trace content are automatically measured again with an appropriate dilution. The detection limit for a 1:1 diluted sample is 2.5 μ g/l. The analytical imprecision of the assay is 2.3%-6.5%.

STATISTICS

To validate the nephelometric assay for β -trace protein in the diagnosis of liquorrhoea the well established electroimmunoassay was used as an external validation criterion. The reference method is coded in the form of a dichotomous variable with the possible outcome 1 (negative) and 2 (positive). The tested method is a continuous variable with a minimum score of 0.2 and a maximum score of 53.9 mg/l. Because the emphasis is on avoiding false positive predictions the critical value for the nephelometric assay was determined for the specificity which reaches 100%. Accordingly, 2x2 contingency tables were determined for 10 different versions of a dichotomised nephelometric variable. The nephelometric β -trace protein value was recoded to 1 (negative) and 2 (positive) by varying the critical value for a negative diagnosis from 1 mg/l to 10 mg/l. Sensitivity and specificity for each critical value are summarised in figure 2. Additionally, χ^2 tests (or Fisher's exact tests) were applied to test for statistical significance of the relation between the two methods.

Results

ELECTROIMMUNOASSAY AS REFERENCE METHOD

Of 140 samples of clinically suspected liquorrhoea, 36 were found to be positive for β -trace protein detected by electroimmunoassay. The

Table 1 Clinical characteristics of patients with CSF leak. The diagnostic procedures that proved the diagnosis are given in bold

Patient No	Age (y)	Sex	Aetiology of CSF leak	Diagnostic procedures	Observation period (months)	Complications
1	68	Female	Unknown	CT , scintigraphy	2	None
2	8	Female	Post-traumatic	CT, cisternography, intrathecal fluorescein, endoscopic surgery	5	None
3	58	Male	Sinusitis sphenoidalis	CT , surgery	0	Meningitis, exitus
4	73	Male	After craniotomy	CT , surgery	7	Meningitis
5	72	Female	After ventriculotomy	CT	31	None
6	83	Male	Frontobasal meningioma	CT , surgery	1,5	None
7	58	Female	Unknown	CT	32	None
8	57	Male	Post-traumatic	CT , surgery	37	None
9	46	Male	After craniotomy	CT , surgery	38	None
10	19	Male	Frontobasal fracture	CT, MRT, intrathecal fluorescein, endoscopic surgery	40	Recurrence which led to revision surgery, none

nephelometric assay for β-trace protein showed 33 positive samples. None of the electroimmunoassay negative samples was positive in the nephelometric assay. Under the premise of a low rate of falsely positively predicted cases (a specificity of 100%) the critical value of the nephelometric assay could be quantified with 6 mg/l or more of protein. If sensitivity was considered simultaneously, the critical value of 6 mg/l was the best choice leading to a sensitivity of 92%. The relation between the electroimmunoassay and the nephelometric assay was highly significant in any chosen critical value (p<0.001).

CLINICAL EVALUATION AS A REFERENCE METHOD

In the group of 52 patients (79 samples), 13 samples from 10 patients were positive for β-trace protein with both methods. In three patients measurements were repeated. In one patient two positive results in two samples were found and in another patient three positive results in five samples were found. The two negative samples of this patient were taken after successful closure of a CSF leak. In the third patient—a child of 8 years with a combined temporal bone and sphenoid sinus fracture—the first sample taken from the nose was positive for β-trace protein. Therefore endoscopic sinus surgery was performed with closure of the sphenoid fracture line. Two postoperative controls from nasal secretion were negative. When the patient developed a bacterial meningitis 8 months later, a sample of nasal secretion was taken and was found negative for β-trace protein. Cranial CT showed signs of fluid in the mastoid cells at the side of the temporal bone fracture. An explorative mastoidectomy and tympanoscopy was performed which showed a dural prolapse with a clear CSF leak. The defect was closed the next day by a temporal approach. It is possible that the nasal secretion from the anterior parts of the nose did not contain any CSF as this time the CSF fistula was located temporally. Although the negative result of this β-trace analysis was due more to inadequate sampling than lack of sensitivity of the method, we judged this analysis as a false negative result. This evaluation gave a sensitivity of 93% for the nephelometric β-trace assay.

In all 10 patients, the clinically suspected liquorrhoea and the positive β-trace protein result could be confirmed by other techniques.

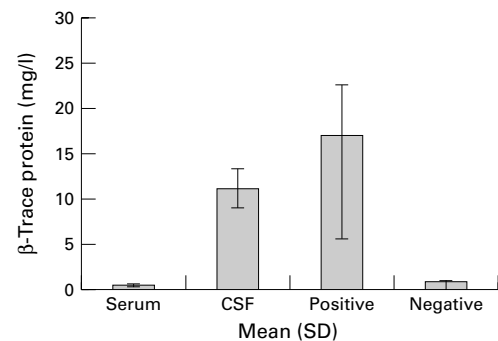


Figure 3 Mean (SD) of β-trace protein concentrations in serum and CSF of patients with tension headache: negative and positive samples.

Table 2 Mean values (mg/l) for serum and CSF β-trace protein measured by nephelometry

First author	Serum β-trace protein	CSF β-trace protein
Felgenhauer ³¹	0.5	17
Tumani ¹⁰	0.5	15
Own results	0.5	11

Table 1 shows the investigations that were performed in the 10 β-trace positive patients. The diagnosis of a CSF leak was proved by CT in seven, by surgical inspection in six, and in one patient by cisternography and intrathecal fluorescein application.

All other samples were negative in the nephelometric and electroimmunoassay.

In none of the β-trace negative patients was a liquorrhoea shown by any imaging technique used or by surgery. Computed tomography was used in 99%, surgical inspection in 49%, MRI in 13%, radionuclide imaging of the subarachnoid space in 3%, and intrathecal fluorescein application in 1% of β-trace protein negative cases. This gave a specificity of β-trace protein detection in the diagnosis of liquorrhoea of 100%.

The mean β-trace protein concentrations measured with the nephelometric assay in serum of 34 healthy volunteers, CSF of 20 patients with tension headache, 107 negative, and 33 positive samples are summarised in figure 3.

Table 2 gives an overview from the literature of mean values for serum and CSF β-trace protein measured by nephelometry.

Discussion

Our results indicate a high sensitivity and specificity of the nephelometric β-trace protein

assay in diagnosing liquorrhoea. This accords with previous findings of a sensitivity of 97.3% and a specificity near 100% using the electroimmunoassay in 101 patients.²² In this study, comparison with the established electroimmunoassay gave a sensitivity of 92% and a specificity of 100%. This was confirmed by the clinical validation, which showed a sensitivity of 93% and a specificity of 100%.

The introduction of the nephelometric assay is a substantial improvement in β -trace protein detection as the method is highly automated; this results in a shorter analysis time of 15 minutes. By comparison, the electroimmunoassay method needs about 3 to 4 hours for the same result. As mentioned above, the rapid diagnosis of liquorrhoea is essential for some physicians, including neurologists, neurosurgeons, and otorhinolaryngologists. Once the diagnosis of liquorrhoea is proved, any effort, including invasive diagnostic procedures or explorative surgery, should be undertaken to localise the site of the leak and to close it before a life threatening meningitis might occur.

From the technical point of view, the nephelometric assay for β -trace protein can be performed with very small amounts of sample (5 μ l are recommended) and is easy to carry out with routine laboratory equipment. False positive results may occur in patients with renal failure who have increased serum β -trace protein concentrations.²⁴ False negative results may occur when contamination is limited (<5%).²⁵ As 10% to 30% of β -trace protein in a sample are bound by gauze swabs, inappropriate sampling may also result in false negative results.²⁵

In our series, the results of β -trace protein measurement in serum and CSF in the control group are in line with previous results.¹⁶ Whereas the serum values were identical with those given in the literature, the CSF values were slightly lower, which might be explained by the lower age and serum/CSF albumin ratio in our control group. The samples negative for β -trace protein showed low mean β -trace protein concentrations, comparable with those measured in the serum of the control group (fig 3). The positive samples showed mean β -trace protein concentrations that were expected for CSF. In a few samples, β -trace protein concentration was unexpectedly high. Together with the broad range of the SD in the group of positive samples, this might reflect the various methods of sampling. These will result in a dilution of the sample containing CSF by other body fluids in most of the cases, but may lead to a higher concentration of the CSF within the sample in isolated cases as well.

Other CSF specific proteins, such as β_2 -transferrin, have been suggested as indicators of liquorrhoea.^{26, 27} However, compared with β -trace protein, the clinical evaluation of β_2 -transferrin showed it to have lower sensitivity and specificity. In early studies, a sensitivity of 79% was found.²⁶ Other findings report a higher sensitivity, of nearly 100%, but a reduced specificity of 95%.²⁷ Reasons for a falsely positive β_2 -transferrin test are underlying hepatic diseases with β_2 -transferrin being detectable in the

serum, also, and allelic variants of the serum transferrin that cannot be distinguished from β_2 -transferrin by the electroimmunoassay.^{28, 29} A further disadvantage of β_2 -transferrin assay is that it is time consuming; it takes 3.5 hours even in its latest improved version.³⁰

A historical marker of liquorrhoea—glucose—has not been considered as it is present abundantly in serum and blood. Even in water-like secretions from the nose or ear it is of limited use as reference values have not yet been established and data on sensitivity and specificity of the method are lacking.

In conclusion nephelometric β -trace protein detection is rapid and highly valid for the diagnosis of a CSF leak and should help to facilitate the management of patients with suspected liquorrhoea.

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HISTORICAL NOTE

Ammon's horn and the hippocampus

The word hippocampus comes from late Latin: hippocampus, derived from the Greek words for a horse+sea monster. In mythology it was a sea horse, having two forefeet, with the body ending in a dolphin's or fish's tail, represented as drawing the vehicle of Neptune the sea God. The earliest use (Oxford English Dictionary) was in 1606, cited as "Drummond of Hawthornden" *Let. Wks.* (1711) 232. "Stately pageants. that of Cheapside was of Neptune on a hippocampus, with his Tritons and Nêreides".

Neurologists recognise it as each of two elongated eminences (hippocampus major and minor) on the floor of each lateral ventricle of the brain; so called from their supposed resemblance to the fish.

With its base in ancient classical history, neuroanatomy provides several metaphors that relate the gods and the brain. One is Ammon's horn. The term *Cornu Ammonis*, or

Ammon's horn, is a well known description of the whorled chambered shells of a fossil genus of Cephalopods. They were once supposed to be coiled snakes petrified, and hence called "snake-stones" from their resemblance to the involuted horn of Jupiter Ammon.

The hippocampus received its name from the Italian Julius Caesar Arantius in the late 16th century.¹ Less than two centuries later, the hippocampus was called Ammon's horn.^{2,3} An early, anatomical use is in the 1742 book of a felicitously named surgeon René Jacques Croissant de Garengéot.⁴ In 1732 Jacques Bénigne Winslow used the term ram's horn. Thus Ammon's horn was probably not in use at this date. Albrecht von Haller, the anatomist, indicated that the term Ammon's horn was already used in a paper of the *Oeconomische Abhandlung* of 1755 (Haller, 1774–7, vol 2 p 507).

The term Ammon's horn is a metaphor that refers to the ram shaped horns⁵ on the head representing the Egyptian God Amun who protected the Pharaoh Taharqa in the temple of Kawa.⁶ Many temples were dedicated to Amun. The Greek form of the name was Ammon, the Libyan Jupiter whom the Greeks identified with Zeus. King David conquered a Jordanian tribe, the Ammonites,

who were descendants of Lot, by the son of his younger daughter.

It is of interest that the related hippocampal commissure together with the crura of the fornix, is sometimes termed the "psalterium" or "lyra Davidis". Psalterium and lyra are both harps.

To add to the confusion, French neuro-anatomists refer to the horn shaped lateral part of the fourth ventricle with its choroid plexus leaving the foramen like a posy of flowers as the "corne d'abondance" (horn of plenty, or cornucopia).

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