

## Evaluation of nephelometry for albumin measurement in serum and cerebrospinal fluid: experiences with an indwelling subarachnoidal catheter system for repetitive cerebrospinal fluid collection in horses

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**Abstract.** The measurement of albumin concentrations in cerebrospinal fluid (CSF) and serum for albumin quotient (AQ) calculations in normal horses was performed by 2 methods: 1) total protein measurement, followed by electrophoresis of the samples to obtain an albumin percentage; and 2) albumin immunoprecipitation quantitated by nephelometry. The results of both methods correlated well, and nephelometry was chosen to determine the albumin concentrations in CSF samples obtained from an indwelling subarachnoidal catheter for daily sampling. Because the use of an indwelling catheter to collect repetitive CSF samples is a novel technique, routine cytological CSF analysis was performed along with daily clinical evaluation to ascertain the well-being of the horses. The catheters were placed in 2 horses for periods of 14 and 17 days. One horse exhibited pleocytosis on cytological evaluation of CSF on 2 occasions for a 1–2-day duration; however, the AQ showed a significant increase on only 1 occasion. The other horse had a normal cell count in CSF but showed 2 sudden changes in the AQ value; however, these values remained within the 95% confidence interval for AQ in horses. Albumin quotient values of the second horse were consistently below the lower range of the confidence interval. Results from this study indicate that nephelometry can be used for albumin determination in serum and CSF samples from horses. Furthermore, an indwelling subarachnoidal catheter system can provide serial CSF samples in horses, thus obviating the need for repetitive centesis for serial CSF sampling.

**Key words:** Albumin quotient; cerebrospinal fluid; horse; nephelometry; repetitive sampling.

### Introduction

Cerebrospinal fluid (CSF) is produced mostly by choroid plexi in the lateral, third, and fourth ventricles of the brain. It flows within the subarachnoidal space of the leptomeninges and exchanges with the extracellular fluid compartment of the central nervous system (CNS) parenchyma.<sup>3</sup> It can therefore be used as a diagnostic sample in CNS disease. Basic CSF clinical pathology includes a cell count, differential, and total protein measurements; however, the current spectrum of measurable CSF parameters has been expanded to meet the clinicians'

needs regarding specific diagnoses.<sup>2</sup> The albumin quotient (AQ), a ratio between the amount of albumin in CSF and serum (albumin [CSF] in g/liter/albumin [serum] in g/liter  $\times$  100), has gained popularity as a parameter for assessing blood-brain barrier (BBB) integrity in horses. A 95% confidence interval (CI) is available for normal AQ values in adult horses (0.6–2.2 for atlanto-occipital CSF samples and 0.7–2.3 for lumbo-sacral CSF samples).<sup>1</sup> Albumin quotient in combination with CSF and serum IgG concentrations (IgG index) helps differentiate between intrathecal immunoglobulin production, a breached BBB, or contamination of CSF with blood during collection.<sup>2</sup> However, results should be interpreted only in combination with a cytological evaluation of CSF. Direct albumin measurements in CSF necessary for AQ calculation are usually not accurate enough to detect the low concentrations in CSF. Until now, albumin concentration in CSF has been determined by total protein measurement followed by electrophoresis of CSF to determine the fraction of albumin from the total protein concentration.<sup>6</sup> Recently, an immunological technique based on detection of albumin-antialbumin immune complexes by nephelometry has been described and evaluated

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for its use in horses (Hammerbacher-Smith KI: 2001, Inaugural dissertation, Ludwig-Maximilians-University, Munich, Germany).<sup>4</sup>

Although repetitive (daily) CSF sampling is difficult to carry out in horses, it can be used to profile AQ or specific cytokine changes during the course of CNS disease along with correlating these changes with clinical (neurological) findings. Repeated CSF sampling is a potentially painful experience for a horse. Moreover, depending on the site chosen for CSF centesis (atlanto-occipital vs. lumbo-sacral), there is a risk of CNS damage. Furthermore, the diagnostic usefulness of repetitive CSF sampling may be compromised by unwanted blood contamination, which can adversely affect the predictive value of the results.<sup>2,3</sup> Although techniques involving (semi)permanent placement of steel cannulas penetrating CSF-containing spaces are described in the rat, the mouse, or in the dog, there is no experience with these techniques in the horse.<sup>5,7</sup>

The objectives of this study were 2-fold: 1) to evaluate nephelometry as an alternative tool for the measurement of albumin concentrations in equine CSF and serum, and 2) to evaluate a short-term indwelling catheterization system of the subarachnoidal space in the horse with a tubing system for daily CSF sampling.

### Materials and methods

The following sets of samples were used to compare nephelometry results with those of protein electrophoresis. Four solutions were prepared by dissolving lyophilized equine albumin in 0.9% NaCl yielding the following concentrations: 0.245, 0.82, 1.225, and 2.45 g/L. Sample group 1 was composed of 5 serum samples from middle-aged crossbred Warmblood horses from which sera were submitted for routine clinical examination. Sample group 2 was composed of paired serum and CSF samples from 9 middle-aged crossbred Warmblood horses of both sexes, with a body weight between 500 and 600 kg. These horses were euthanized with an intravenous overdose of pentobarbital for various reasons not related to CNS pathology. A third group of sample pairs was obtained from the subarachnoidal catheter study and was evaluated by nephelometry only. For this group, 2 institution-owned horses (horses A and B) were used. Horse A was a 17-yr-old crossbred mare (body weight: 510 kg), and horse B was an 18-yr-old Warmblood gelding (body weight: 620 kg). After successful placement of the catheter, both horses were stall confined but were hand walked twice daily starting on day 3 after catheter placement. From 3 days before catheter placement through the experimental period to 3 days after the removal of the subarachnoidal catheter, these horses underwent daily clinical examinations. During this period, venous blood samples in EDTA from both horses were submitted for white blood cell (WBC) counts with an automated hematology analyzer.<sup>a</sup> As soon as the indwelling

subarachnoidal catheter was in place, serum and CSF were collected once daily. The indwelling catheter<sup>b</sup> was placed in the 2 standing, sedated horses by a lumbo-sacral approach following routine CSF collection procedures and precautions.<sup>3</sup> An 18-gauge, 15-cm Tuohy needle<sup>b</sup> (stiletted spinal needle with special bevel orientation) was advanced with its opening oriented cranially until CSF could be aspirated through the needle. Then, a guide-wire-enforced 20-gauge closed-end catheter (80 cm long) was advanced cranially through the Tuohy needle into the subarachnoidal space toward the caudal aspect of the third lumbar vertebra. The needle and guide wire were removed, and the external part of the catheter was affixed to the skin followed by careful wrapping. The external end of the tubing was closed with a special locking system, included in the kit. A rubber injection cap<sup>b</sup> was attached onto the end of the locking system. At the end of the experimental period, the catheters were removed. For reasons unrelated to this study, both horses were euthanized at 2 mo (horse A) and 3 wk (horse B) after removal of the catheters. A complete postmortem examination was performed. All experiments on live animals were performed with permission from and following the guidelines of the Animal Welfare Committee (DEC) of Utrecht University, The Netherlands.

All serum samples were divided into 2 equal portions and stored frozen at  $-20^{\circ}\text{C}$ . For group 2 horses, blood samples for serum collection were drawn before euthanasia. Serum samples from the indwelling catheter study were collected once daily and within minutes after CSF collection. Cerebrospinal fluid in group 2 horses was collected by atlanto-occipital centesis immediately after euthanasia. Twenty milliliters of CSF was collected, of which the first 5 ml was discarded. In horses from the subarachnoidal catheter study, 7 ml of CSF was aspirated once daily through the tubing system with 2 syringes. The first 2 ml of CSF collected in syringe 1 was discarded to avoid the submission of CSF that had been in the tubing system since the last sampling. One-milliliter aliquots of CSF were placed into EDTA tubes and used for cytological examination. The CSF samples collected from the 9 (group 2) horses had to meet criteria of  $<10$  WBC/ $\mu\text{l}$  and  $<50$  red blood cells (RBC)/ $\mu\text{l}$  to remain in the study. Cerebrospinal fluid samples from the catheter study with  $>10$  WBC/ $\mu\text{l}$  were submitted for a WBC differential. For this purpose, a cytopspin preparation<sup>c</sup> with 200  $\mu\text{l}$  of CSF ( $40 \times g$ , 10 minutes) was prepared, followed by routine May-Grünwald Giemsa staining. All CSF samples were centrifuged at  $1,000 \times g$  for 5 min to remove any cells and debris, and the supernatant was immediately stored in duplicate in 1.5-ml cryovials at  $-20^{\circ}\text{C}$ .

Albumin measurements in serum and CSF were performed by 3 methods: 1) direct measurement of albumin in serum and CSF (albumin direct); 2) measurement of the total protein concentration, followed by multiplication with the percentage albumin determined by electrophoresis (total protein [g/liter]  $\times$  albumin % = albumin [g/liter]) (albumin electrophoresis); and 3) immunoprecipitation of albumin, quantitated by nephelometry. Albumin electrophoresis was considered the reference standard according to earlier publications.<sup>1,6</sup> For the albumin-direct method,

**Table 1.** Results of correlation calculations for various comparisons.\*

	<i>r</i>	<i>R</i> <sup>2</sup>	<i>y</i>
Calibration curve (BN II) vs. albumin solutions (0.245, 0.82, 1.225, and 2.45 g/liter of lyophilized equine albumin in NaCl [0.9%])	0.999	0.999	1.08x - 0.02
Serum albumin ( <i>n</i> = 5) in g/liter	0.999	0.999	1.11x - 4.2
Albumin direct (BCG method) vs. albumin electrophoresis			
Serum albumin ( <i>n</i> = 9) in g/liter	0.902	0.87	0.904x - 2.07
Albumin electrophoresis vs. nephelometry (Fig. 1)			
CSF albumin ( <i>n</i> = 9) in g/liter	0.984	0.968	0.964x - 0.016
Albumin electrophoresis vs. nephelometry (Fig. 2)			
AQ ( <i>n</i> = 9) in % CSF/serum electrophoresis vs. CSF/serum nephelometry (Fig. 3)	0.984	0.967	0.975x + 0.209

\* BN II = Behring Nephelometer; BCG = bromocresol green; CSF = cerebrospinal fluid; AQ = albumin quotient.

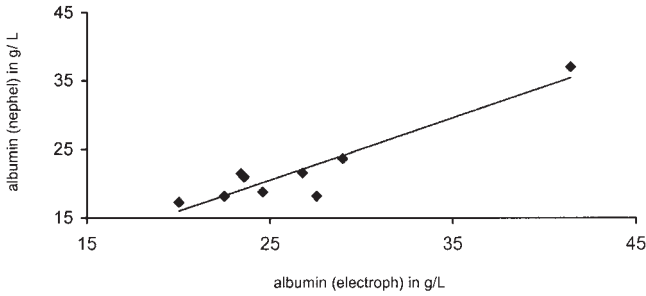
a bromocresol green<sup>d</sup> colorimetric method was used and the reaction product was measured at 600 nm wavelength in a Beckman CX7 autoanalyser.<sup>c</sup> Serum total protein was measured by using a biuret reaction<sup>e</sup> in which colored complexes of peptide copper were measured at 560 nm wavelength after 465 sec. Cerebrospinal fluid samples were analyzed with Microprotein (M-TP)<sup>e</sup> in the autoanalyser<sup>c</sup> with a pyrogallol red molybdate complex to produce a purple color that can be measured at 600 nm wavelength. Electrophoresis was performed with standard agarose SPE-gels<sup>e</sup> according to manufacturer's instructions. Serum samples were diluted (1 : 5) in a barbital buffer (pH 8.6)<sup>e</sup> before application on the gel, whereas CSF samples were applied undiluted. During each analysis a normal protein electrophoresis control<sup>c</sup> was included. The various fractions from electrophoresis were reported as percentages.

For the nephelometry method, a Behring Nephelometer BN II<sup>f</sup> was used as previously described (Hammerbacher-Smith KI: 2001, Inaugural dissertation, Ludwig-Maximilians-University, Munich, Germany). The principle of this assay is based on the detection of albumin-antialbumin antibody precipitates that cause mie-scattering in the light beam of a 840-nm light-emitting diode.<sup>4</sup> Lyophilized equine albumin<sup>g</sup> was dissolved in 0.9% NaCl to a final concentration of 38 g/liter. This stock solution served as a standard, and 10-fold dilutions of the stock were automatically prepared by the BN II. The antibody used for precipitation was an antihuman albumin polyclonal antibody.<sup>h</sup> The amount of light scattered is proportional to the amount of precipitate that is formed; the albumin fraction in samples was determined by interpolation to values obtained with the standard dilutions. All nephelometric measurements were completed in triplicate. To control the measurements of the BN II, 4 albumin control solutions with concentrations of 0.245, 0.82, 1.225, and 2.45 g/L were prepared by dissolving lyophilized equine albumin<sup>g</sup> in 0.9% NaCl. The 4 control solutions were blinded from the technician, who measured albumin by using nephelometry. Because 1 solution could not be recovered from the nephelometer, only 3 of these samples were analyzed by albumin electrophoresis. The 5 serum samples from group 1 horses underwent albumin measurements by 2 methods: albumin direct and albumin electrophoresis. The 9 serum-CSF pairs (group 2 samples) were analyzed by albumin electrophore-

sis and nephelometry. All sample pairs from the subarachnoidal catheter study were analyzed by nephelometry only. Results of nephelometry and albumin electrophoresis were compared, and a correlation coefficient (*r*) and its coefficient of variation (*R*<sup>2</sup>) were calculated by using Microsoft Excel.<sup>i</sup> Results were plotted in a scattergram, and a trend line was fitted through the results. The formula for the trend line (*y* = *ax* ± *b*) providing the slope (*ax*) and intersection (*b*) with the *y*-axis was calculated by using Microsoft Excel.

## Results

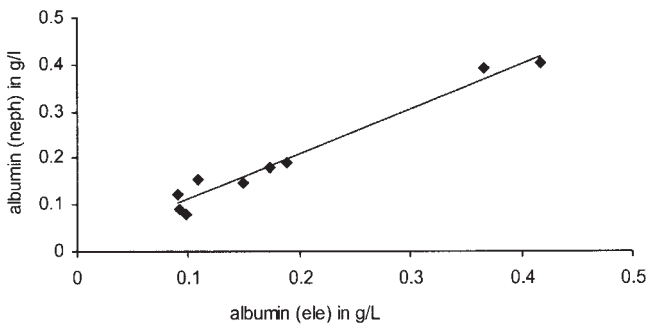
Albumin concentrations were successfully determined by nephelometry in CSF and serum samples. The various steps of albumin measurement and validation by using the different techniques of albumin quantitation are listed in Table 1, which also lists the correlation coefficient, the coefficient of variation, and the mathematical formula for the trend line as calculated for the techniques that were compared by using the same set of samples. The concentrations of the prepared equine albumin solutions were accurately determined by nephelometry (*r* = 0.999) and slightly less accurately by albumin electrophoresis (*r* = 0.982). The discrepancy was found in the microprotein measurement and not in the electrophoresis. Albumin-direct results correlated well with those of albumin electrophoresis for the serum samples (sample group 1, *r* = 0.999). Direct albumin measurements in CSF were not possible because the low albumin concentration in CSF is below the detection limit of this test. Therefore, an AQ could be calculated only based on the results of albumin electrophoresis or nephelometry. The individual trend line formulas for albumin measurements in serum and CSF show that nephelometry slightly underestimates albumin concentrations because the slope of the trend line for both calculations is <1. This effect is slightly more pronounced with the albumin measurements in serum (*y* = 0.904x - 2.07) (Fig. 1) than in CSF (*y* = 0.964 - 0.016) (Fig. 2). The



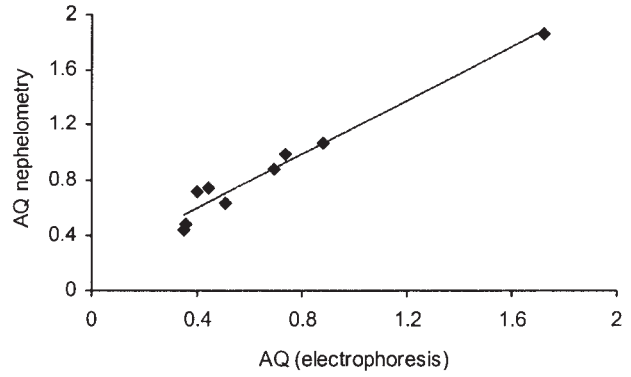
**Figure 1.** Correlation of albumin measurements (in g/liter) in serum by electrophoresis (x-axis) and by nephelometry (y-axis) in CSF-serum pairs obtained from 9 horses. Correlation coefficient  $r = 0.902$ ; coefficient of variation  $R^2 = 0.87$ , trend line  $y = 0.904x - 2.07$ .

AQ is minimally affected by this underestimation (Fig. 3). However, because of the more pronounced effect in the serum albumin measurements, the AQ calculated by nephelometry is slightly higher than the AQ calculated by albumin electrophoresis (Table 1).

Subarachnoidal catheter placement in both horses was achieved without difficulties. The horses appeared normal during daily examinations up to 3 days after the catheter was removed. Objective parameters, such as rectal temperature and peripheral WBC counts, were normal throughout the experiment. Cerebrospinal fluid aspiration through the tubing was easily achieved in horse B, whereas the tubing system was obstructed on 1 occasion in horse A. The average AQ, calculated from the nephelometric results of serum and CSF analysis, was  $0.595 \pm 0.058$  for horse A, which is below the lower end of the CI (95% CI for AQ determined from lumbo-sacrally obtained CSF samples: 0.7–2.3).<sup>1</sup> The calculated AQ from subsequent samples obtained from horse B remained within this CI with a mean AQ of  $1.215 \pm 0.149$  but showed a sudden increase in AQ on day 3 (Fig. 4a). From days 13–14 there was a second,

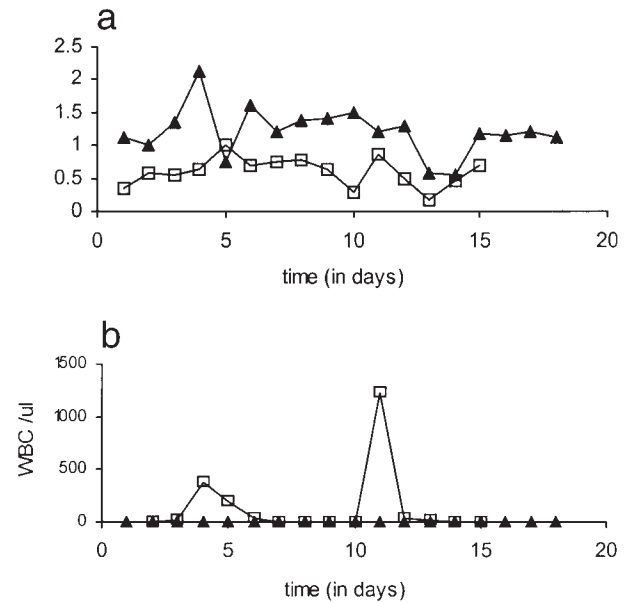


**Figure 2.** Correlation of albumin measurements (in g/liter) in CSF by electrophoresis (x-axis) and by nephelometry (y-axis) in CSF-serum pairs obtained from 9 horses. Correlation coefficient  $r = 0.984$ , coefficient of variation  $R^2 = 0.968$ , trend line  $y = 0.964x - 0.016$ .



**Figure 3.** Albumin quotient calculations (albumin [CSF] in g/liter/albumin [serum] in g/liter  $\times 100$ ) with albumin measurements obtained by electrophoresis (x-axis) or by nephelometry (y-axis). Correlation coefficient  $r = 0.984$ , coefficient of variation  $R^2 = 0.967$ , trend line  $y = 0.975x + 0.209$ .

unexplainable sudden decrease in AQ in this horse. These changes in AQ were not preceded or accompanied by changes in the other measured parameters. Horse A experienced a mild to moderate pleocytosis (400 and 1,200 WBC/ $\mu$ l; >90% polymorphonucleated [PMN] cells) on days 4 and 11 (Fig. 4b). The first increase in CSF cell count occurred after hand walking on day 3. The day before the second episode of pleocytosis, the catheter was occluded and subsequently flushed with 2 ml of heparin-containing (50 IU/ml) 0.9% NaCl. On both occasions cell counts in CSF returned to normal within 24 hours. Despite



**Figure 4.** Longitudinal results (time in days) from 2 horses with a subarachnoidal catheter system in place (lumbo-sacral approach). Horse A (open squares) and horse B (closed triangles). **a** Albumin quotient (albumin [CSF] in g/liter/albumin [serum] in g/liter  $\times 100$ ). **b** Albumin measurements done by nephelometry and WBC concentration in CSF.

the pleocytosis in the CSF in horse A, peripheral blood leukocyte counts and rectal temperature were not affected by these developments. Surprisingly, the AQ changes in horse A were less dramatic despite the leukocyte influx when compared with horse B, where the leukocyte count in CSF remained normal throughout the entire period. Hand walking did not affect CSF cell counts in horse B, and catheter patency was maintained throughout the study. A pain reaction was not noticed during removal of the catheters. Both catheters appeared clean and undamaged on macroscopic inspection. Histopathological examination of the lumbar spinal cord showed no anomaly in horse A, whereas mild chronic inflammatory changes were observed in the dura at the penetration site of the catheter in horse B.

### Discussion

Nephelometry can be a valid alternative for conventional albumin measurements in serum and CSF. It was demonstrated that an antibody against human albumin is able to detect albumin in equine samples. This is not surprising because of the conserved molecular structure of albumin among mammalian species. Immunoprecipitation and quantitation of equine albumin with a human polyclonal antibody was demonstrated both in prepared solutions with a known amount of dissolved equine albumin and in natural samples (serum and CSF). Albumin determination via nephelometry showed greater variation in serum than in CSF samples when compared with albumin electrophoresis. Albumin is an important carrier molecule in blood, and the greater variation of albumin determination in serum than in CSF may be explained by the presence of more interfering substances (mainly proteins and globulins) in serum, which may have blocked antibody-specific determinants. Calculated AQ values were not significantly different regardless of the method of albumin determination in CSF and serum. Cerebrospinal fluid and serum albumin concentrations should be measured by the same technique to obtain valid AQ results.

Nephelometry has the advantage of being a fast method, which requires only a small sample volume (<1 ml). Additionally, the technique can be used to measure immunoglobulin concentrations in CSF and serum, with the possibility of calculating an immunoglobulin index in the same samples.<sup>2</sup> Neither nephelometry nor albumin electrophoresis is capable of distinguishing between normal albumin content and albumin influx into CSF-containing spaces as a result of a breached BBB (trauma), subarachnoidal hemorrhage, or hemorrhage resulting from the CSF sampling technique. In such situations, a total cell

count (including WBC and RBC) may be required to interpret the AQ results correctly. The use of an indwelling subarachnoidal tubing system can reduce the risk of blood contamination in serial CSF samples. However, because this catheter system is "foreign" material, when used in an immunocompetent animal it can cause irritation and an inflammatory response at the insertion site. Because CSF flows in a cranial-to-caudal fashion, it could be advantageous to advance the catheter tip more cranially to obtain a sample least influenced by local tissue factors or inflammation at the catheter insertion site. However, care should be taken not to advance the catheter too far cranially. Because the subarachnoidal space is wider at the lumbar spinal cord intumescence than in other parts of the spinal cord,<sup>3</sup> advancing the tip of the catheter too far cranially might close the meninges too tightly around the catheter tip, thus preventing effective CSF collection. Shortly after insertion of a subarachnoid catheter, a mild inflammatory response can be expected. In the rat model, a 7-day waiting period is necessary before sampling to allow initial inflammation to cease and for healing of the breach in the BBB.<sup>5</sup> According to the changes in the AQ value near day 13 in both horses, it is possible that the period around day 13 could represent the time at which the BBB breach was completely healed in these horses. However, even after healing of the breach in BBB, accidental micromovements of the catheter at the insertion site may further affect the composition of CSF. Cytological evaluations of CSF samples were performed only when >10 WBC/ $\mu$ l were found. The influx of WBCs was predominantly caused by polymorphonuclear leukocytes (PMN). It was not evaluated whether an earlier shift from a mononuclear cell population to a majority of PMN cells had already occurred in the CSF samples. However, early cytological evaluation is strongly recommended because it could represent an even earlier indicator of inflammation or indicate problems at the catheter insertion site. Therefore, daily cell counts and protein measurements in CSF are important parameters to monitor in any experiment where CSF is collected with the indwelling catheter described in this study.

In the present study, a novel technique for collection of CSF samples and determination of AQ in a time-efficient manner was described and validated in the horse. The technique needs to be validated for CSF samples obtained from horses with CNS disease. The subarachnoidal catheter system was maintained for up to 17 days. However, on the basis of the gained experience, once the catheter is placed it may be maintained for longer than 17 days at the same site. Cerebrospinal fluid analysis (differential

cell count and AQ calculation), clinical parameters, and peripheral blood WBC counts are recommended daily to monitor any catheter-related complications.

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- c. Cytospin 2, Shandon, Zeist, The Netherlands.
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