Detection of CSF Leakage by Isoelectric Focusing on Polyacrylamide Gel, Direct Immunofixation of Transferrins, and Silver Staining, Freek W.C. Roelandse,* Nico van der Zwaard, Jan H. Didden, Jenny van Loon, and John H.M. Souverijn (Dept. of Clin. Chem., Leiden Univ. Medical Centre, Albinusdreef 2, 2333 ZA Leiden, The Netherlands; *author for correspondence: fax + 31/71/5266753, e-mail roelandse@rullf2.medfac.leidenuniv.nl)

Leakage of cerebrospinal fluid (CSF) from the subarachnoid space into the nasal or aural cavity creates a pathway for life-threatening central nervous system infection. Detection of the leakage can be cumbersome [1]. In former days the identification and localization of CSF leakage was almost entirely dependent upon the clinical history and methods involving intrathecal injection of radioactive material or a dye. Furthermore, various chemical tests, such as determination of glucose, protein, and electrolytes, performed on the secreted material have been advocated for the differentiation between CSF leakage and other secretions [2]. The injection methods are risk-bearing for the patient and the above methods yield a considerable chance of false-positive or false-negative results [3]. Nowadays O-sialotransferrin, also named β2-transferrin, asialotransferrin, or the τ fraction, is an accepted marker protein for the detection of CSF in excretions from the nose or the ear, or from head or neck wounds. O-Sialotransferrin is not strictly unique to CSF. The protein can also be demonstrated in human aqueous humor [4] and in perilymph fluid [5–7]. It is also found in the blood of patients with chronic liver diseases [8, 9], with an inborn error of glycoprotein metabolism [10], and with a genetic variant of transferrin [8, 11, 12]. The protein can be detected by direct immunofixation of transferrins after agarose electrophoresis (high-resolution electrophoresis, HRE) [3, 13] or after blotting [1, 14, 15]. The method we used up to now was the agarose/immunofixation method according to Zaret et al. [3]. We used the Paragon High Resolution Electrophoresis kit (HRE kit, Beckman Instruments) and an anti-transferrin reagent (Beckman Array® System, Beckman Instruments). One disadvantage of this fast method is the high protein concentration that is necessary for use: The secretion extract must be concentrated to a protein amount of 10 g/L. Therefore small samples cannot be analyzed. Furthermore, the location of the O-sialotransferrin band in secretions can be displaced, compared with the location of the O-sialotransferrin band in a control CSF. This phenomenon hampers detection. An admixture of large quantities of blood (hemoglobins) disturbs the pattern because of β2-transferrin [3].

Our currently described method improves the detection of O-sialotransferrin in the case of minor CSF leakages and even in small blood-containing samples. The described method is not meant to be a stat method. O-Sialotransferrin (pI 5.9) and other transferrin isoforms, such as monosialo- (pI 5.8), disialo- (pI 5.7), and trisialotransferrin (pI 5.6), are easily recognized. The method is suited for small samples, requires less protein, is sensitive, and gives more information compared with the agarose electrophoresis methods. Because in rare cases O-sialotransferrin can occur in blood, the necessity of involving a serum sample from the same patient for comparison is obvious.

In detail, the procedure is as follows: Nose and ear secretions from patients suspected of having a CSF leakage are absorbed with Merocel® tampons, cotton swabs, or gauze. Next the material is extracted with a small amount of distilled water. After centrifugation of the extract at 1900g for 10 min at room temperature, the total protein concentration of the supernatant is measured (Hitachi 911 analyzer, Boehringer Mannheim). Blood samples to prepare serum are obtained through venipuncture. After centrifugation at 1900g for 10 min at room temperature, the total protein concentration of the serum is measured (Hitachi 747 analyzer, Boehringer Mannheim). Control CSF consists of a CSF sample that shows a clear fraction of the O-sialotransferrin by isoelectric focusing (IEF). Extract supernatant, serum, and control CSF are stored at –20°C before analysis. If required, extract supernatant and control CSF are concentrated in Fugisep® centrifuge concentrators (type PES 10, Intersep Filtration Systems) to a total protein amount of 250 mg/L. Serum and extract supernatant with a total protein amount of >250 mg/L are diluted to 250 mg/L with a 1:2 mixture of saline and distilled water. pH focusing experiments are performed on a Multiphor® II electrophoresis kit with a 2103 power supply (Pharmacia-LKB) equipped with a Kryostat WK5 cooling unit (Colora) adjusted to 8°C. Polyacrylamide gels are ready-to-use Ampholine® PAG plates for IEF, pH range 3.5–9.5 (Pharmacia Biotech). Sample volume is 20 μL. Samples of patients’ sera are placed adjacent to the prepared extract. Control CSF is incorporated in each assay. The power supply is limited to a maximum of 1500 V and 50 mA. Total focusing time is 90 min. After pH focusing the PAG plate is (gel-side down) placed on top of a 1-mm layer of a 50-fold dilution in distilled water of transferrin antibody (A0061, Dako Corp.) in a plexiglass tray. Incubation is for 60 min at 37°C. After the incubation several washings with distilled water are applied (overnight). Silver staining is performed at room temperature with a 1 g/L silver nitrate solution as coloring reagent for 30 min, a developer reagent containing 30 g of sodium carbonate and 500 μL of formalin per liter for 4–10 min (dependent on the visual band intensity/background ratio), a 480 g/L citric acid solution as stopping reagent for 10 min, and a 50 g/L glycerol solution as preserving reagent for 30 min (all chemicals for the silver staining are purchased from Merck). Finally the PAG plate is covered with a cellophane preserving sheet (Pharmacia Biotech), which is
fixed with glass strips on a glass plate. The PAG plate is dried for 5 h at 60 °C. This method gives excellent results in recognizing O-sialotransferrin in secretions. In Fig. 1 a comparison with the agarose/immunofixation assay is demonstrated for five patients. All samples were extractions from cotton swabs in which the secretion was collected. All the specimens were contaminated with blood. Patient 1 shows a positive finding for O-sialotransferrin with the currently described method, but the test is negative with the agarose/immunofixation assay. Patients 2 and 4 show positive findings with both methods. Patient 3 is clearly positive with the currently described method, but only slightly positive with the agarose/immunofixation assay (hardly visible in the Figure). Patient 5 is negative with both methods.

Of 43 samples, we found 11 to be positive and 26 to be negative with both the agarose/immunofixation assay and with the IEF method; 6 samples were positive with the IEF method, but negative with the agarose/immunofixation assay. None of the samples was positive with the agarose/immunofixation assay when a negative result was obtained with the IEF method. From these data we can conclude that the currently described method is more sensitive than the agarose/immunofixation assay: With the proposed method we found 17 samples positive for CSF, whereas with the former method only 11 samples were positive. Not only O-sialotransferrin is seen, but also other transferrin isoforms such as monosialo-, disialo-, and trisialotransferrin. This low sialic acid transferrin pattern is characteristic for CSF (admixture). We thank the staff of the department for their cooperation in the production of this manuscript.
The deleterious biological effects of lead ingestion are well established. There has been a growing concern that ingestion of certain oyster shell-containing calcium supplements and antacids might contribute to an increased risk of lead poisoning in individuals who consume these products [1]. Consumers of calcium supplements and calcium antacids, especially pregnant and lactating women, their unborn fetuses, infants, and small children would thus be exposed to a serious and documented risk of reproductive and developmental harm. Because calcium supplements are invariably prescribed in the prevention and treatment of osteoporosis, elderly osteoporotic individuals may also be at increased risk for lead toxicity. Because the skeleton represents the major repository of lead in the human body, containing 90% or more of total lead content [2], we reasoned that measurement of skeletal lead concentration in osteoporotic individuals before and after calcium supplementation would disclose whether there was significant lead accumulation from administration of calcium supplements.

The patient population was composed of 11 women and three men with a mean age of 60 years (range 38–76). All patients had osteoporosis as evidenced by reduced vertebral bone mass, histologic evidence of osteoporosis, and at least one vertebral fracture. All patients and controls were involved in research protocols approved by the Institutional Review Board on the Use of Human Subjects. A transcortical iliac crest bone biopsy was obtained from each patient after obtaining informed consent and before beginning calcium citrate therapy (400 mg of calcium twice daily as Citracal®, Mission Pharmacal). A second bone biopsy was procured on the opposite side after completing, on average, 4.75 years of calcium therapy (range 1–9 years). Four healthy men (mean age 35 years, range 20–53) who participated in a different protocol without calcium supplementation and who also underwent transcortical iliac crest bone biopsy 12 weeks apart served as methodological controls.

Bone biopsies were dehydrated and embedded in methylmethacrylate for sectioning and histologic analysis. Upon completion of bone histology, a 100-μm longitudinal section was cut from each biopsy with a low-speed wafering saw. These sections were then ashed overnight in tared platinum crucibles at 600 °C and the ash weight of bone (representing the mineral phase) was determined. Ashed bone specimens (5–20 mg ash weight) were dissolved in 1 mL of 5% nitric acid and diluted to 2 mL with water from a MilliQ (Millipore) water purification system. In addition, sections of methylmethacrylate that contained no bone specimen were also removed and subjected to the same procedure to ensure that the embedding material contained no significant amounts of lead. Bone lead was quantified via electrothermal atomic absorption spectrometry. The analytical equipment was a Varian SpectraAA 20 equipped with a graphite tube assembly (GTA 96) electrothermal oven (Varian Instruments). A calibration curve was prepared over the range of 0.2–1 ng of elemental lead in 2.5% nitric acid with a total injection volume of 25 μL. The sample was dried at 100 °C (ramp 5 s, hold 10 s), ashed at 400 °C (ramp 10 s, hold 8 s), and atomized at 2000 °C (5 s). The absorbance was measured at 283.3 nm. A deuterium background correction was used because the above-mentioned spectrophotometer was not equipped with Zeeman correction capabilities. Samples (25 μL) were assayed directly without further additions. This methodology gave a detection limit of 0.05 ng of lead when processed and assayed as described above. The intraassay CV was 3.9% when determined on three specimens assayed 15 times each (range 1.6–6.5%). The interassay CV was 4.5% when determined for three different concentrations on five different days (range 2.5–5.6%). Assessment of method accuracy yielded an average recovery of 107% (range 97–116%). Preliminary studies in a few specimens demonstrated comparable results for either direct addition or by calibrator additions. Thus, all results were obtained by the method of direct analysis.

Table 1 summarizes the results. For all 14 patients, mean bone lead increased nonsignificantly (P = 0.203) from 3.3 ± 1.8 to 4.2 ± 1.8 ng/mg bone ash. All mean values were within reported normal values of 1–8 ng/mg bone ash when performed by electrothermal atomic absorption spectrometry [3–5]. Variability of lead in the iliac crest was observed to be minimal on the basis of sampling from contralateral sites 12 weeks apart in the four healthy subjects. It is interesting to note that pre- and postmean

<table>
<thead>
<tr>
<th>Group</th>
<th>Age</th>
<th>Bone lead (ng/mg bone ash)</th>
<th>Pre</th>
<th>Post</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 4)</td>
<td>35 ± 14</td>
<td>1.5 ± 0.7</td>
<td>1.5 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>Patients (n = 14)</td>
<td>60 ± 12</td>
<td>3.3 ± 1.8</td>
<td>4.2 ± 1.8</td>
<td></td>
</tr>
</tbody>
</table>

*Calcium treatment was 400 mg of calcium twice a day as calcium citrate for a mean treatment period of 4.75 years in patients only.

Values expressed as mean ± SD.