HPLC method for measurement of purine nucleotide degradation products in cerebrospinal fluid

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We describe a convenient method for the separation and quantification of xanthine, hypoxanthine, and uric acid in 20 µL of cerebrospinal fluid (CSF) with use of HPLC and ultraviolet detection. The analysis is performed on a Sepharon SGX C₁₈ column and the elution system consists of potassium phosphate buffer, pH 5.1, with 20 mL/L methanol. The lower limit of detection was 4 pmol for hypoxanthine and xanthine and 6 pmol for uric acid. Analytical recoveries of purine metabolites ranged from 98.6% to 102.9%. The intra- and interassay CVs were <3%. The applicability of the method is illustrated with the determination of micromolar concentrations of xanthine, hypoxanthine, and uric acid in CSF samples obtained from 113 patients with various neurological disorders.

INDEXING TERMS: xanthine • hypoxanthine • uric acid • neurological disorders • ROC curve analysis

Biochemical analysis of cerebrospinal fluid (CSF) by HPLC may contribute to the diagnostic process and to the optimization of therapy in neurological and psychiatric disorders.

Degradation products of purine nucleotides—xanthine and hypoxanthine—which are accumulated during hypoxia in brain tissue as well as in CSF, have been shown to be markers of central nervous system hypoxia [1–4] and to provide therapeutic guides in hydrocephalus [5, 6]. Several methods for quantification of xanthine and hypoxanthine have been reported, but none of these methods has been modified for routine assay in a small volume of human CSF. Here we report a new, simple, and sensitive HPLC method for quantifying degradation products of purine nucleotides in CSF. Optimization of separation conditions was based on the analysis of effects of organic modifier, ionic strength, and pH on retention times of measured substances. Suitability of this method for routine clinical use was verified by determination of oxopurine concentrations in CSF samples from 113 patients with various neurological disorders.

Materials and Methods

Reagents. Hypoxanthine and xanthine were obtained from Sigma (St. Louis, MO), uric acid from Merck (Darmstadt, Germany), xanthine oxidase (EC 1.2.3.2) and uricase (EC 1.7.3.3) from Boehringer Mannheim (Mannheim, Germany), and methanol and potassium dihydrogen phosphate from Lachema (Brno, Czech Republic). All chemicals were reagent grade; water was deionized and triply distilled.

Apparatus. The HPLC unit consisted of a pump (Model 2150; LKB, Bromma, Sweden), an injector with a 20–181 µL loop (Model 7125; Rheodyne, Cotati, CA), a variable-wavelength ultraviolet detector (Model 2151; LKB) with a deuterium lamp, and a strip-chart recorder (Model SE 120; BBC Goerz Metrawatt, Vienna, Austria). The isocratic separation was carried out on two in-series connected Sepharon SGX C₁₈ columns (300 mm length, particle size 7 µm; Laboratorní prístroje, Praha, Czech Republic).

Chromatographic conditions. The eluent, potassium phosphate buffer (0.06 mol/L, pH 5.1, 20 mL/L methanol), was filtered (nitrocellulose filter, 0.45 µm; Millipore, Bedford, MA) and degassed by shaking under reduced pressure before use. The chromatograph was operated isocratically at ambient temperature with a flow rate of 0.5 mL/min. Absorbance of the eluent was measured at 254 nm, with the detector sensitivity set at 0.08 A full scale.

Specimen handling. CSF samples were obtained by lumbar puncture, and an aliquot of each specimen was immediately centrifuged, decanted, and stored frozen at −20 °C until analysis. Just before analysis the samples were thawed in a waterbath at 20 °C.

Specification and calculations. Hypoxanthine, xanthine, and uric acid calibrators were prepared by appropriate mixing and serial

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2 Nonstandard abbreviations: CSF, cerebrospinal fluid; CT, computed tomography; MRI, magnetic resonance imaging; and AUC, area under the curve.

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water dilution of stock solutions, each containing 1 mmol/L in 10 mmol/L NaOH, to cover the following concentration ranges: 5–100 μmol/L for uric acid, and 0.5–50 μmol/L for hypoxanthine and xanthine. The working calibrators were stored in 1-mL aliquots at −20 °C. The concentration of each analyte was calculated by a regression equation describing the appropriate calibration curve.

Imprecision and recovery. Within-day imprecision was tested by measuring seven 0.5-mL samples taken from one CSF specimen during the day. To assess analytical recovery, 20 μL of a calibration solution was added to 80-μL aliquots of seven different CSF specimens. The calibration solution contained hypoxanthine and xanthine, 20 μmol/L each, and uric acid, 50 μmol/L. The specimens were analyzed before and after the addition.

Patients. CSF samples for the determination of oxopurines were obtained from patients diagnosed as having thromboembolic focal cerebral ischemia (n = 10, age 62.3 ± 2.8 years), multiple sclerosis (n = 11, 41.5 ± 1.9 years), aneurysmal subarachnoid hemorrhage (n = 27, 53.1 ± 4.0 years), polyneuropathy (n = 10, 46.3 ± 4.6 years), or prolapse of the intervertebral disc (n = 40, 47.3 ± 1.5 years). The clinical diagnosis was based on careful neurological examination and other necessary standard procedures [computed tomography (CT), magnetic resonance imaging (MRI), myelography, CSF analysis]. The selected patients had neither primary nor secondary hyperuricemia. The reference group was composed of healthy volunteers (n = 11, age 44.4 ± 3.0 years) in whom basic CSF fluid variables (cell count, total protein, albumin, glucose, chloride) were within physiological ranges. The CSF collection procedure was approved by the Institutional Ethics Committee and was in accordance with the Helsinki Declaration II.

Statistics. Results are presented as arithmetic mean ± SD. Data were analyzed with standard statistical methods (goodness-of-fit test, F-test), and the Mann–Whitney test was used for statistical comparison of differences in the means. Correlation analysis was performed by means of Spearman rank correlation test. A receiver–operating characteristic (ROC) curve analysis was used to characterize the diagnostic accuracy of xanthine, hypoxanthine, and uric acid [7]. Parametric ROC curves and areas under ROC curves were calculated with the ROC analysis PC software kindly given by C.E. Metz (Department of Radiology, University of Chicago, Chicago, IL).

Results

Figure 1a depicts a representative chromatogram of a mixture of uric acid, hypoxanthine, and xanthine. The mobile phase was optimized with respect to the concentration of analytes in CSF, resolution of neighboring maxima in CSF, and to the time of analysis. Fig. 1b represents a chromatogram of a CSF sample. Optimal composition of the mobile phase was determined by checking three variables: pH, concentration of methanol, and concentration of KH₂PO₄. Varying the latter in the concentration range 10–150 mmol/L altered the retention and resolution of oxopurines minimally (Fig. 2A). By contrast, the methanol content in the mobile phase had a profound effect, as shown in Fig. 2B. There was a concentration-dependent decrease in retention as well as resolution of all purine metabolites. The effects of the mobile phase pH on the retention of purine metabolites are presented in Fig. 2C. One can observe that the pH affects mainly retention of the uric acid.

Hypoxanthine, xanthine, and uric acid in the chromatogram were identified by comparing the retention times for the pure substances (Table 1) and by the enzymatic peak shift technique [8]. Chromatograms of CSF samples pretreated with xanthine oxidase or uricase are shown in Fig. 3. Data on imprecision and recovery are presented in Table 1. The detection limits, defined as two times the baseline noise, were 4 pmol for hypoxanthine and xanthine and 6 pmol for uric acid. Calibration curves were linear for all oxopurines within the range tested, i.e., up to 50 μmol/L for hypoxanthine and xanthine, and up to 100 μmol/L for uric acid.

In Table 2 the concentrations of xanthine, hypoxanthine, uric acid, total protein, and albumin in CSF of healthy individuals and in patients with various neurological diseases are presented. Uric acid concentrations in CSF were significantly increased in all patients tested except the patients with multiple sclerosis. Increased concentrations of xanthine and hypoxanthine were observed only in patients with thromboembolic focal cerebral ischemia. In this group of patients, statistical analysis revealed close positive correlation between uric acid and the

![Fig. 1. HPLC chromatograms of uric acid (UA), hypoxanthine (HYP), and xanthine (XAN) in a calibration solution and in a CSF sample chromatographed on the Separon SGX C₁₈ column.](image-url)
Fig. 2. Effect of (A) potassium phosphate buffer concentration (pH 4.7), (B) methanol concentration, and (C) pH on the retention of uric acid, hypoxanthine, and xanthine, expressed as capacity ratio ($k'$).

Table 1. Retention time (RT), analytical recovery (AR), and within-run and day-to-day imprecision (CV) of hypoxanthine, xanthine, and uric acid in CSF (n = 7).  

<table>
<thead>
<tr>
<th></th>
<th>RT, min</th>
<th>AR, %</th>
<th>CV, %</th>
<th>Within-run</th>
<th>Day-to-day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uric acid</td>
<td>8.5</td>
<td>100.6 ± 0.7</td>
<td></td>
<td>1.7</td>
<td>2.0</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>12.5</td>
<td>102.9 ± 4.2</td>
<td></td>
<td>2.0</td>
<td>2.7</td>
</tr>
<tr>
<td>Xanthine</td>
<td>13.8</td>
<td>98.6 ± 2.8</td>
<td></td>
<td>2.6</td>
<td>2.9</td>
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</table>

concentrations of total protein and albumin ($r = 0.750$ and $0.762$, $P < 0.05$) and negative correlation between hypoxanthine concentration and concentrations of total protein and albumin ($r = -0.883$, $-0.881$, $P < 0.05$).

The diagnostic sensitivity and specificity of oxopurine concentrations in CSF for focal cerebral ischemia, aneurysmal subarachnoid hemorrhage, multiple sclerosis, polyneuropathy, and prolapse of intervertebral disc were evaluated by ROC curve analysis. Fig. 4 represents the ROC curves for xanthine, hypoxanthine, and uric acid for patients with cerebral ischemia. The corresponding calculated area under the curve (AUC) is 0.9907 ± 0.0148 for xanthine, 0.7800 ± 0.1131 for hypoxanthine, and 0.8859 ± 0.0906 for uric acid if specificity is based on the group of healthy individuals (Fig. 4A), but much lower (AUC$_{\text{xanthine}} = 0.9203 ± 0.0304$, AUC$_{\text{hypoxanthine}} = 0.7691 ± 0.0942$, AUC$_{\text{uric acid}} = 0.6898 ± 0.0998$) if the reference group consists of patients with other neurological diseases (Fig. 4B). The diagnostic accuracy of all three variables in other neurological diagnoses was very low (AUC < 0.8).

**Discussion**

Despite significant advances in methodology, the techniques available for measuring purine degradation products in CSF [1, 8-10] are somewhat limited and none is entirely suitable for routine clinical use. In this paper we describe an isocratic, rapid, highly sensitive, and reproducible HPLC method for the determination of hypoxanthine, xanthine, and uric acid in CSF. The technique requires only 20 µL of native CSF and can yield both

Fig. 3. Identification of peaks in CSF sample by enzyme peak shift method.  
(a) Injection of 20 µL of CSF pretreated with uricase; (b) injection of 20 µL of the same CSF sample after treatment with xanthine oxidase. Inverted chromatogram in (a) represents chromatogram of the same sample, but detector sensitivity was 10 times lower.
Table 2. CSF concentrations (mean ± SD) of xanthine, hypoxanthine, uric acid, total protein, and albumin.

<table>
<thead>
<tr>
<th></th>
<th>Xanthine μmol/L</th>
<th>Hypoxanthine μmol/L</th>
<th>Uric acid μmol/L</th>
<th>Total protein g/L</th>
<th>Albumin ± g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>2.00 ± 0.38</td>
<td>2.74 ± 0.60</td>
<td>18.96 ± 6.54</td>
<td>0.27 ± 0.06</td>
<td>0.26 ± 0.06</td>
</tr>
<tr>
<td>CI</td>
<td>3.14 ± 0.46***</td>
<td>3.79 ± 1.14*</td>
<td>50.84 ± 29.02**</td>
<td>0.35 ± 0.16</td>
<td>0.35 ± 0.12</td>
</tr>
<tr>
<td>SAH</td>
<td>3.28 ± 1.47**</td>
<td>3.94 ± 2.15</td>
<td>41.34 ± 26.14**</td>
<td>0.36 ± 0.23</td>
<td>0.37 ± 0.26</td>
</tr>
<tr>
<td>MS</td>
<td>1.94 ± 1.15</td>
<td>2.98 ± 0.89</td>
<td>27.99 ± 19.32</td>
<td>0.34 ± 0.18</td>
<td>0.26 ± 0.04</td>
</tr>
<tr>
<td>PN</td>
<td>1.89 ± 0.56</td>
<td>2.14 ± 0.82</td>
<td>43.25 ± 19.40*</td>
<td>0.46 ± 0.29</td>
<td>0.31 ± 0.08</td>
</tr>
<tr>
<td>LIS</td>
<td>2.10 ± 0.69</td>
<td>2.95 ± 0.86</td>
<td>32.68 ± 20.55*</td>
<td>0.46 ± 0.23*</td>
<td>0.37 ± 0.13*</td>
</tr>
</tbody>
</table>

C, healthy volunteers; CI, patients with thromboembolic cerebral ischemia; SAH, patients with aneurysmal subarachnoid hemorrhage; MS, patients with multiple sclerosis; PN, patients with polyneuropathy; and LIS, prolapse of the intervertebral disc.

*P < 0.05, **P < 0.01, ***P < 0.001.

Fig. 4. ROC curves of CSF concentrations of xanthine, hypoxanthine, and uric acid for patients with focal cerebral ischemia vs (A) healthy individuals and (B) patients with other neurological diagnoses.

Table: qualitative and quantitative information about the profile of the purine degradation products in <30 min.

Optimization of the method was done by analysis of effects of mobile phase pH, ionic strength, and content of organic modifier on retention and resolution. We found that pH has the most profound effect. The differences in retention characteristics of oxopurines associated with changes of mobile phase pH were due to different dissociation constants (pKᵦ) of analytes. The pKᵦ of hypoxanthine is ~8.80–8.94 and the pKᵦ of xanthine 7.44–7.53 [11], and the retention was unchanged up to pH 6.0. The pKᵦ value for uric acid is 5.40–5.78 [11], and there was a close relation between decreased retention and increased ionization at higher pH values.

The reference values of purine metabolites in CSF obtained by our HPLC method are comparable with those reported by previous authors using HPLC [2, 3, 12]. Comparison of CSF concentrations of xanthine and hypoxanthine in patients with thromboembolic cerebral ischemia and in patients with other neurological disorders suggests that increased concentrations of these metabolites could have clinical usefulness as a marker of ischemic damage in the central nervous system. Repetitive measurement of CSF concentrations of oxopurines in combination with imaging techniques (CT, MRI) could provide additional information regarding extent and progression of brain tissue damage. Clinical applicability was confirmed by the ROC analysis, which revealed high diagnostic accuracy of increased CSF concentration of xanthine for thromboembolic focal cerebral ischemia (AUC > 0.9).

In summary, the use of this method provides a rapid approach to the examination of CSF concentrations of hypoxanthine, xanthine, and uric acid in various neurological disorders. The sensitivity is sufficient to detect as little as 4 pmol of these substances. The precision is acceptable, not exceeding 3.0%, and analytical recoveries were 98.6–102.9%. Given the absence of any cumbersome specimen pretreatment procedure, no internal standard is needed to obtain good precision and accuracy.

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References


