Simultaneous Liquid-Chromatographic Determination of Hypoxanthine, Xanthine, Urate, and Creatinine in Cerebrospinal Fluid, with Direct Injection

Frank Niklasson

In this method for simultaneously determining hypoxanthine, xanthine, urate, and creatinine in cerebrospinal fluid, centrifuged sample is directly injected on a reversed-phase liquid-chromatographic column. On elution with potassium phosphate buffer the compounds are quantified by their absorbance at 260 nm. Random error (CV) was between 1.2 and 3.4% and analytical recoveries were 99–104% at physiological concentrations.

Additional Keyphrases: chromatography, reversed-phase

With the development of "high-performance" liquid chromatography (HPLC) it has become possible to separate and quantify most intermediates of purine metabolism. Since the original demonstration of the separation of purine bases and nucleotides on reversed-phase HPLC (1), several authors have modified the separation system and the specimen pretreatment in adopting this technique to such different biological materials as blood, urine, and tissue extracts (for a review see 2). Analyses for hypoxanthine and xanthine in cerebrospinal fluid (CSF) have until now been performed by enzymatic methods (3), enzyme–oxygen electrode methods (4) or thin-layer chromatography (5). Unfortunately, these methods are nonspecific or too insensitive, or both. Urate has ordinarily been determined by enzymatic methods, and creatinine spectrophotometrically by the Jaffe reaction.

The absence of the enzyme xanthine oxidase in brain (6) indicates that xanthine rather than urate is the end product of brain purine metabolism. An initial observation of a concentration gradient across the blood–brain barrier for hypoxanthine and xanthine with the higher values in the CSF (Niklasson, unpublished) opened up the possibility that the CSF concentrations of these purines reflect the intrathecal purine metabolism rather than the blood concentration. I wished to investigate this thoroughly in patients suffering from certain pathological conditions of the central nervous system, and also to evaluate the potential diagnostic and prognostic value of measuring purine compounds in CSF. To do so, I developed the present HPLC method. It is accurate, precise and sensitive enough to detect even subnormal concentrations of hypoxanthine, xanthine, urate, and creatinine in the same isocratic run. Furthermore, no specimen pretreatment is needed, except for centrifugation.

Materials and Methods

Materials

Reagents. Hypoxanthine and xanthine were obtained from Sigma Chemical Co., St. Louis, MO 63178. Uric acid and creatinine were obtained from E. Merck, Darmstadt, F.R.G. The enzymes xanthine oxidase (EC 1.2.3.2), uricase (EC 1.7.3.3), creatinase (EC 3.5.2.10), creatine kinase (EC 2.7.3.2), and pyruvate kinase (EC 2.7.1.40), and ATP were obtained from Boehringer Mannheim GmbH, Mannheim, F.R.G. The reversed-phase packing material, μBondapak C18, was from Waters Associates, Milford, MA 01757. The chemicals were of reagent grade and the water used to prepare the buffer was de-ionized and doubly distilled in an all-glass system.

Apparatus. The HPLC system consisted of a two-plunger pump (Constamatic III; Laboratory Data Control, Riviera Beach, FL 33404), a Rheodyne loop injector (Model 7125) with a loop size of 20 μL, a variable-wavelength ultraviolet-detector (Spectromonitor III, Laboratory Data Control) and a Servogor 120 ink writer. The analytical columns were 300 × 3.9 mm containing μBondapak C18, packed in the laboratory with the aid of a Haskel DST-150 air-driven fluid pump, by use of the upward slurry technique (7), with the support suspended in methanol (packing pressure 35 MPa).

Chromatographic conditions. The eluent, potassium phosphate buffer (0.2 mol/L, pH 6.6), was ultrafiltered and degassed by shaking it under reduced pressure before use. The chromatograph was operated isocratically at ambient temperature, with a flow rate of 1.0 mL/min. The absorbance at 260 nm was monitored and the sensitivity setting was usually 0.01 A full scale.

Procedures

Specimen handling. Measured volumes of CSF were obtained by lumbar puncture, and an aliquot of each specimen was centrifuged, decanted, and stored frozen at −20 or −70 °C until analysis. Before analysis, the specimens were thawed at about 20 °C in a waterbath, then kept in an ice bath until injected. Minor erythrocyte contamination did not affect the results if the intact cells could be removed by centrifugation. If hemolysis occurred, hypoxanthine increased, being the main degradation product of purines in erythrocytes.

Calibration standards and calculations. Four working standards were prepared by appropriate mixing and serial dilution of stock standard solutions, each containing 1.00 mmol/L (in distilled water for creatinine and in 10 mmol/L NaOH for the others), to cover the following concentration ranges: 12.5–100 μmol/L for creatinine, 6.25–50 μmol/L for urate, and 0.625–10 mol/L for hypoxanthine and xanthine. The working standards were stored in 1-mL portions at −20 °C. A new set of working standards was thawed on each analytical occasion and kept in the ice-bath until injected. The concentrations were calculated from the regression lines of the standards' peak heights vs concentrations, by using a minicalculator.

Identification. Hypoxanthine, xanthine, urate, and creatinine in CSF were identified by comparing their retention with the retention of pure standards and by their degradation on addition of enzymes. To do this, I added 1 μL of undiluted xanthine oxidase solution and (or) uricase solution to 200 μL of CSF for 10 min at 22 °C before injection. For complete degradation of creatinine I used three enzymes in coupled enzyme reactions (8). The following reagents were added to 200 μL of CSF: 1 μL of undiluted solutions of creatinase and pyruvate kinase, 2–3 μg of creatine kinase, 3.5.2.10), creatine kinase (EC 2.7.3.2), and pyruvate kinase (EC 2.7.1.40), and ATP were
and about 20 µg each of ATP, phosphoenolpyruvate, and magnesium chloride. The reaction was allowed to proceed for 20 min at 22 °C, after which 20 µL of the reaction mixture was injected on the HPLC column.

Imprecision and recovery. Within-day imprecision was tested by dividing one CSF specimen into 0.5-mL portions in eleven tubes and making one injection from each of the tubes during the day. Day-to-day imprecision was calculated by analyzing a solution of known concentrations on 25 occasions during a period of three months. In the recovery experiment, 20 µL of a standard solution was added to 80-µL aliquots of seven different CSF specimens. The standard solution contained hypoxanthine and xanthine 20 µmol/L each, urate 50 µmol/L, and creatinine 100 mol/L. The specimens were analyzed before and after the addition.

Results

Figure 1 illustrates four typical HPLC chromatograms obtained by the present method. The separation of pure standards is shown in 1a and the elution pattern of some components in CSF in 1b. The result of enzymatic degradation of hypoxanthine and xanthine by xanthine oxidase is presented in 1c. Finally, the elimination of the urate peak on addition of uricase is clear in 1d. Creatinine was degraded in a similar way after addition of the proper enzymes.

The retention of each analyte was influenced by the pH of the eluent (Figure 2). Optimal separation of the actual analytes from other compounds present in CSF was at pH 6.6.

Hypoxanthine, xanthine, urate, and creatinine have absorbance maxima at 250, 270, 293, and 236 nm, respectively, at pH 6.6. Monitoring absorbance at 260 nm is thus not optimal for any of the analytes. However, owing to their presence in different concentrations in CSF, all four could be detected without changing attenuation during most of the runs. This means that the sensitivity of the method for the respective analytes can be increased by simply changing the wavelength of the detector.

Data on imprecision and recovery are presented in Tables 1 and 2. The limits of detection, defined as twofold the baseline noise, were 3.2 pmol (0.16 µmol/L) for hypoxanthine and xanthine, 53.9 pmol (0.2 µmol/L) for urate, and 7.8 pmol (0.39 µmol/L) for creatinine (injection volume 20 µL).

Standard curves were linear for all four substances within the range tested, i.e., up to 50 µmol/L for hypoxanthine and xanthine and 100 µmol/L for urate and creatinine.

Discussion

Specimens of biological origin usually have to be deproteinized before injection onto HPLC columns, precipitation and ultrafiltration being the methods most commonly used for this (9). Because CSF can be regarded as an "ultrafiltrate of plasma" (protein content normally <0.5 g/L), it was tempting to see if the CSF could be injected onto the column directly after centrifugation. Such a technique in fact allows separation of several purine metabolites and creatinine on reversed-phase HPLC columns. Given the absence of any cumbersome specimen-pretreatment procedure, no internal standard was needed to obtain good precision and accuracy. It should, however, be possible to improve the precision further by using an electronic integrator to measure peak area, owing to the difficulty of measuring peak heights with less error than ±0.5 mm. This would correspond to variations of 2.5-1.6% when peak heights are 20-30 mm, which often is typical for hypoxanthine and xanthine.

The differences in retention for the four analytes, when the pH of the eluent was changed, were due to their different dissociation constants (pKₐ). The pKₐ of hypoxanthine is about 8.80-8.94 (10) and the retention was unchanged until pH 7 (about 1% is dissociated at that pH). The pKₐ values for xanthine and urate are about 7.44-7.53 and 5.40-5.78, respectively (10), their retention decreasing with increasing ionization at higher pH. Similar results have previously been obtained with the packing material Nucleosil 5µ C8 (Macherey Nagel, F.R.G.) in columns of similar dimensions (11). However, these columns deteriorated quickly, probably because of the higher pH (7.2) needed to achieve complete separation.

Because of the concentration gradient between cisternal CSF and lumbar CSF for many analytes (12), the concentration of an analyte in a CSF specimen obtained by lumbar
We have recently reported reference values for hypoxanthine, xanthine, and urate in CSF (11). The CSF concentrations of hypoxanthine and xanthine were higher than corresponding values in plasma obtained by HPLC techniques (14, 15, and my own unpublished results). The concentration of urate in CSF, on the other hand, was only about 5% of that in serum, in good agreement with results of enzymatic determinations (3). Measurement of CSF urate concentrations in pathological states could thus be a potential marker of blood–brain barrier dysfunction.

The method presented here is superior to methods that do not allow separate estimations of hypoxanthine and xanthine concentrations (4) and also to other HPLC methods designed for only one of the oxypurines (16); valuable information about the pathophysiological mechanisms in several disorders of the central nervous system will be provided by assaying both. My coworkers and I have so far been able to demonstrate correlations between CSF oxypurines and certain symptoms in depressed patients (13); correlations of CSF oxypurines and the CSF monoamine metabolites 5-hydroxyindoleacetic acid and homovanillic acid (17), and disturbances of the intrathecal purine metabolism due to hypoxia in cardiovascular disorders (11). Hypoxanthine concentrations in CSF reportedly are increased in bacterial meningitis (16).

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References
13. Ägren H, Niklasson F, Hallgren R. Brain purinergic activity linked with depressive symptomatology: Hypoxanthine and xan-

puncture may depend on the specimen volume obtained. On the other hand, if constant volumes are obtained from different individuals, one finds a correlation between height (i.e., the position in the spinal canal) and concentration of some analytes. From the following two observations—(a) a negative correlation between patient height and CSF concentrations of hypoxanthine and xanthine (13) and (b) higher concentrations in one reference population, from whom 18 mL of CSF was withdrawn (Niklasson and Lindström, to be published), than in another, from whom 12-mL samples were withdrawn (11)—one can suppose that a cisternal–lumbar concentration gradient also exists for hypoxanthine and xanthine. It therefore is important to standardize the sampling procedure and to specify the CSF volume taken for these analytes when determining reference values and when comparing reference populations with patient populations.

Table 1. Imprecision Estimates Within Day (a) and Between Day (b)

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SD</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypoxanthine</td>
<td>2.33 (2.48)</td>
<td>0.057 (0.090)</td>
<td>2.4 (3.6)</td>
</tr>
<tr>
<td>Xanthine</td>
<td>1.93 (2.51)</td>
<td>0.065 (0.083)</td>
<td>3.4 (3.3)</td>
</tr>
<tr>
<td>Urate</td>
<td>11.25 (25.00)</td>
<td>0.19 (0.41)</td>
<td>1.7 (1.7)</td>
</tr>
<tr>
<td>Creatinine</td>
<td>47.3 (49.9)</td>
<td>0.58 (1.30)</td>
<td>1.2 (2.6)</td>
</tr>
</tbody>
</table>

*a* = 11.

*b* = 25.

Table 2. Analytical Recovery of Purines and Creatinine Added to Seven Different CSF Specimens

<table>
<thead>
<tr>
<th>Added, nmol</th>
<th>Mean</th>
<th>SD</th>
</tr>
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<tbody>
<tr>
<td>Hypoxanthine</td>
<td>0.4</td>
<td>103.5</td>
</tr>
<tr>
<td>Xanthine</td>
<td>0.4</td>
<td>98.8</td>
</tr>
<tr>
<td>Urate</td>
<td>1.0</td>
<td>100.5</td>
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<tr>
<td>Creatinine</td>
<td>2.0</td>
<td>101.7</td>
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</tbody>
</table>
Test-Request Patterns for Clinical Chemistry in a British and a Canadian Renal Dialysis Unit

Mary D. Gardner¹ and A. Ralph Henderson²

We recorded, during four months, the total number of routine clinical chemistry tests requested for patients with end-stage renal failure who were on regular maintenance hemodialysis during the study: 20 in the Renal Dialysis Unit of a British and 20 in a Canadian teaching hospital. The pattern of tests ordered was substantially the same in each unit, but the frequency of testing was not. The total number of tests (and tests per patient per month) was 1616 (20.2) for the British and 6939 (86.7) for the Canadian unit (significant at p < 0.001). This study suggests a significant difference between the two countries in the utilization of laboratory services, and supports our earlier findings on total clinical chemical workloads in hospitals.

Additional Keyphrases: laboratory test utilization - economics of laboratory operation

We previously showed that Canadian clinicians request up to eight times as many tests as do their British counterparts (1, 2). Because the patient mix in each country and in each hospital was not entirely similar, however, we decided to compare the requesting patterns from a more circumscribed group of patients. Treatment of patients undergoing regular hemodialysis for end-stage renal failure is unlikely to vary significantly in different countries, and we have compared the clinical chemistry test workload in Glasgow Royal Infirmary (GRI) and in University Hospital (UH), London, Canada, by comparing the tests requested by the Renal Dialysis Units in each hospital. Both are teaching hospitals in the tertiary-care category as defined by Mechanic (3).

Materials and Methods

We monitored the number of individual tests done on each patient during four months (April through July, 1981). Patients entering or leaving the dialysis program during this period were not included. Twenty patients from each hospital were dialyzed regularly all during this four-month period; similar dialysis schedules were used in each Unit.

Both Units sent "routine bloods" to the laboratory for the tests listed in Table 1, according to a set protocol established by the physicians in each Renal Unit. Funding for these protocols arises from the operating budget of each hospital and is not influenced by, or subject to, any external agency. At GRI the physician can order from a menu of tests on the Request Form; at UH, entire profiles or individual constituent tests of that profile can be ordered on the Request Form (4). The methodologies in use in each hospital have been described (2, 4). The requested patterns in GRI and UH were similar, although GRI added determinations of aluminum and parathyrin (which UH subsequently added in 1982), and UH added glucose, γ-glutamyltransferase, osmolality, and the isoenzymes of lactate dehydrogenase.

We used the Mann–Whitney U Test to test the significance of the monthly data between hospitals (5).

Results and Discussion

Although the pattern of tests is similar, the frequency of requesting is not. The total number of tests done during the study period was 1616 in GRI and 6939 in UH. This amounts to a mean of 20.2 tests per patient per month (range 15.5–36.3) for GRI and 86.7 (58–178) for UH, a significant difference (p < 0.001). The weekly laboratory workload in UH is roughly equivalent to the monthly workload in GRI.

The expected workload figures are 17–19 tests per patient per month for GRI and 62 for UH. The actual workload is therefore an increase of 12.2% and 39.8%, respectively, over the expected workload. The surveillance of the three diabetic patients in the UH group added an additional 178 glucose estimations. Omitting the glucose tests from the UH test numbers reduces the actual workload at UH to 84.5 tests per patient per month. There were no diabetic patients in GRI Renal Dialysis Unit at the time of this study.

At GRI, priority (UH: "stat") tests account for only a small proportion of the "unexpected" tests. In the four-month period GRI had five priority requests (or 15 tests): two requests for electrolytes, urea, and creatinine (one from each of two patients), and three requests for potassium from a digitalized patient. At UH, there were 542 "stat" tests (i.e.,