Liquid-Chromatographic Measurement of Purine Nucleotides in Blood Cells

Roselyne Boulieu, Claude Bory, and Colette Gonnet

In this anion-exchange “high-performance” liquid-chromatographic method of analysis for purine nucleotides, the nucleotides are separated with high efficiency and selectivity on a weak anion exchanger (Hypersil APS 2, 3-μm particle size) by elution with a gradient of eluent pH and concentration. Applying this method to analysis for these compounds in human blood cells, we determined them in a patient with adenosine deaminase deficiency who was treated with a bone-marrow transplantation, finding that the transplantation did not entirely correct the patient’s abnormalities of purine metabolism.

Additional Keyphrases: gradient chromatography, ion-exchange • adenosine deaminase deficiency • purine metabolism • bone-marrow transplantation • nucleotide profile

Nucleotides play an important role in the regulation of cellular functions. The intracellular pool of nucleotides is altered in cells of leukemic patients (1) and in cells of patients with a purine enzyme defect associated with immunodeficiency diseases (2–4). More recently, investigations have focused on the role and toxicity of deoxyribonucleotides in severe combined immunodeficiency disease.

Currently, nucleotides are usually measured by “high-performance” liquid chromatography (HPLC), commonly with strong anion exchangers (1, 5–10). However, nucleotides have also been separated by reversed-phase chromatography, with (11–15) or without (16–18) ion-pairing agents.

Here we describe an anion-exchange HPLC method involving use of a new weak anion exchanger, developed for use in analysis for purine ribonucleotides and deoxyribonucleotides, with high efficiency and selectivity. We applied the described method to analysis for these compounds in cell samples and determined purine nucleotides in a patient with adenosine deaminase deficiency, who had been treated with a bone-marrow transplantation.

Materials and Methods

Apparatus. We used an SP 8000 “high-performance” liquid chromatograph (Spectra Physics, Orsay les Ulis, France) equipped with a fixed-wavelength ultraviolet detector set at 254 nm and a Valco-valve loop injector. The column (15 cm × 4.6 mm, i.d.) was slurry packed, under a pressure of 49 × 10⁶ Pa, with Hypersil APS 2 of 3-μm particle size (Shandon, Ergany, France). In this weak anion exchanger, the amino group with anion-exchange properties is bonded to the silica matrix via an aliphatic propyl spacer.

Reagents. Nucleotides were purchased from Sigma Chemical Co., St. Louis, MO. The ammonium dihydrogen phosphate used in HPLC eluents was either “Gold Label” (99.999%) from Aldrich Chemical Co., Milwaukee, WI, or was prepared by mixing phosphoric acid (“Suprapur”; Merck, Darmstadt, F.R.G.) with “Suprapur” ammonia solution to obtain the desired molarity. Perchloric acid was from Merck.

Chromatographic conditions. For the chromatographic elution we used two NH₄H₂PO₄ buffers. The low-concentration buffer, 30 mmol/L, was adjusted to pH 3.5 with phosphoric acid; the high-concentration buffer was 700 mmol/L, pH 2.8. After an isocratic period of 2 min with the low-concentration buffer, we applied a linear gradient from 0 to 300 mL of the high-concentration buffer per liter over the next 20 min, then kept the eluent at 30/70 (by vol) high-concentration/low-concentration buffer for 18 min. Under these conditions, the buffer concentration increased from 30 to 230 mmol/L. The flow rate was 1.2 mL/min.

Sample collection and treatment. Blood samples were collected in a heparinized tube. In accordance with our previous findings, each sample was centrifuged without delay, to prevent metabolic changes for plasma left in contact with the erythrocytes (15, 20). The plasma was decanted and stored, after deproteinization, for analysis as for nucleotides (21). After removing the leukocytes and the upper layer of erythrocytes, we rapidly deproteinized 500 μL of the remaining erythrocytes by adding 60 μL of 350 g/L perchloric acid and centrifuging (10 min, 1500 × g, 4 °C). After centrifugation, we removed the supernates and adjusted them to pH 6–7 with 5 mol/L sodium hydroxide. The extracts were stored at −20 °C until analysis. Lymphocytes were isolated from freshly drawn heparinized blood on a one-step Ficoll–Isopaque gradient (22) by centrifugation at 700 × g for 35 min. After washing the lymphocytes three times with isotonic saline (NaCl, 9 g/L), we resuspended the cell pellet (~1–5 × 10⁶ cells/mL) in isotonic saline and removed an aliquot for cell counting. The lymphocyte suspension was treated in the same way as the erythrocytes.

Results and Discussion

Chromatographic separation. Nucleotides are negatively charged solutes with hydrophobic interactions. Thus their resolution can be based on either ionic or hydrophobic interactions. These compounds can be well resolved by using an aminopropylsilyl bonded-phase HPLC weak anion-ex-
change support (Hypersil APS 2, 3, μm), which exhibits both ionic and hydrophobic properties.

The ion concentration of the buffer and the pH of the mobile phase strongly affect the ion-exchange interactions. Figure 1a shows that the phosphate ion concentration in the eluent significantly influences the retention of the nucleoside mono-, di-, and triphosphates. An increase in the ionic strength from 30 to 600 mmol/L decreases the retention of these compounds.

The pH of the mobile phase affects mainly the column capacity ratios (k') at low buffer ion concentration. Figure 1b shows that k' for the nucleoside monophosphates varies widely with the pH of the mobile phase at 30 mmol of NH₄H₂PO₄ per liter, whereas at 300 mmol/L the nucleoside diphosphates show only minor variations. Figure 1b also shows that the nucleoside monophosphates should be best resolved at pH 3,5, the diphosphates at pH 3.0.

From these data, we devised a gradient elution for pH and a NH₄H₂PO₄ concentration to separate the purine nucleotides. Figure 2 shows the chromatogram of adenine, guanine, hypoxanthine, xanthine ribonucleoside mono-, di-, and triphosphates and of the mono-, di-, and triphosphate of adenine deoxyribonucleosides. The retention time for nucleotides depends on the number of phosphate groups, the monophosphates being eluted first, followed by the diphosphates and triphosphates.

The microparticulate, chemically silica-bonded weak anion exchanger we used provided columns of high efficiency, to as many as 9000 theoretical plates for a 15-cm column, as measured with adenosine 5'-monophosphate (AMP).

The phosphate buffer gradient elution that is commonly used in ion-exchange chromatography induces baseline drifts because of ultraviolet-absorbing impurities in the buffer (10). To decrease baseline drift, we used either "99.999% Gold Label" NH₄H₂PO₄ or a buffer prepared by neutralization of "Suprapur" H₃PO₄ with "Suprapur" NH₄OH. In this anion-exchange chromatographic system with the phosphate buffer gradient elution mode noted, the purine nucleotides can be separated in 40 min.

Recovery, linearity, and detection limit of adenine and guanine nucleotides. Analytical recovery of the nucleotides of interest as determined by analyzing for nucleotides added to a 200 g/L albumin solution, which was then deproteinized and neutralized, was 96% (SD = 5%; n = 4 for each compound). The detection limit was 5 pmol for the monophosphates, 7 pmol for diphosphates, and 20 pmol for triphosphates. Calibration curves were linear up to 100 μmol/L.

Applications. We applied this HPLC method to analysis for purine nucleotides in human cells.

In healthy subjects, the major purine nucleotides in erythrocytes and lymphocytes are the mono-, di-, and triphosphates of adenine and guanine, with the adenine ribonucleotides predominating (Table 1). The deoxyribonucleotides of adenine are undetectable in erythrocytes and in lymphocytes. Figure 3 illustrates the nucleotide profile for erythrocytes of an apparently healthy subject.

We also determined the concentrations of purine nucleotides in cells from a patient with adenosine deaminase (EC 3.5.4.2) deficiency, who had been treated by bone-marrow transplantation 10 years previously. This deficiency is associated with severe combined immunodeficiency disease (4), and leads to an increase of deoxyadenosine 5'-triphosphate (dATP) and deoxyadenosine 5'-diphosphate (dADP) in erythrocytes and lymphocytes, and an accumulation of adenosine and deoxyadenosine in plasma, erythrocytes, and urine (3, 23). This patient has recovered normal immunological functions after the bone-marrow transplantation. To

![Fig. 1. Influence of the mobile phase composition on column capacity ratios (k'): (a) effect of phosphate ion concentration at pH 3.0, (b) effect of pH at buffer ion concentration of 30 and 300 mmol/L.](image-url)
Fig. 2. Chromatographic separation of purine nucleotides
Peaks: 1, adenosine 5'-monophosphate (AMP); 2, 2'-deoxyadenosine 5'-monophosphate (dAMP); 3, guanosine 5'-monophosphate (GMP), xanthosine 5'-monophosphate (XMP); 4, inosine 5'-monophosphate (IMP); 5, adenosine 5'-diphosphate (ADP); 6, 2'-deoxyadenosine 5'-diphosphate (dADP); 7, guanosine 5'-diphosphate (GDP); 8, xanthosine 5'-diphosphate (XDP); 9, inosine 5'-diphosphate (IDP); 10, adenosine 5'-triphosphate (ATP); 11, 2'-deoxyadenosine 5'-triphosphate (dATP); 12, guanosine 5'-triphosphate (GTP); 13, xanthosine 5'-triphosphate (XTP); 14, inosine 5'-triphosphate (ITP)

Table 1. Nucleotide Concentrations in Erythrocytes and Lymphocytes from Six Healthy Subjects and from an Adenosine Deaminase-Deficient Patient after Bone-Marrow Transplantation

<table>
<thead>
<tr>
<th>Concn in erythrocytes, μmol/L</th>
<th>AMP</th>
<th>ADP</th>
<th>dADP</th>
<th>GDP</th>
<th>ATP</th>
<th>dATP</th>
<th>GTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient</td>
<td>10</td>
<td>170</td>
<td>4.5</td>
<td>13</td>
<td>1178</td>
<td>14</td>
<td>33</td>
</tr>
<tr>
<td>Control subjects</td>
<td>8 (2)*</td>
<td>155 (7)</td>
<td>ND</td>
<td>16 (4)</td>
<td>1170 (260)</td>
<td>ND</td>
<td>32 (8)</td>
</tr>
<tr>
<td>Concn in lymphocytes, nmol/10^6 cells</td>
<td>0.15</td>
<td>0.6</td>
<td>ND</td>
<td>0.2</td>
<td>1.8</td>
<td>ND</td>
<td>0.6</td>
</tr>
<tr>
<td>Patient</td>
<td>0.10 (0.06)*</td>
<td>0.7 (0.3)</td>
<td>ND</td>
<td>0.3 (0.2)</td>
<td>4.2 (2.1)</td>
<td>ND</td>
<td>0.8 (0.5)</td>
</tr>
<tr>
<td>Control subjects</td>
<td>0.10 (0.06)*</td>
<td>0.7 (0.3)</td>
<td>ND</td>
<td>0.3 (0.2)</td>
<td>4.2 (2.1)</td>
<td>ND</td>
<td>0.8 (0.5)</td>
</tr>
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*Mean (and SD). ND, not detectable. dAMP and dGMP were not detectable in either sample.

Fig. 3. Chromatogram of purines in an erythrocyte sample from a healthy subject
Injection volume: 10 μL. Peaks: 1, AMP; 2, nicotinamide adenine dinucleotide (NAD); 3, ADP; 4, GDP; 5, ATP; 6, GTP
evaluate the efficacy of bone-marrow transplantation in rectifying its purine metabolism abnormalities, we determined the concentrations of adenine deoxyribonucleotides (dATP and dADP) in its erythrocytes and lymphocytes. The chromatogram of the patient’s erythrocyte sample (Figure 4) shows that dATP and dADP were present at 14.0 and 4.5 \(\mu\)mol per liter of packed cells, respectively, whereas both compounds were undetectable in erythrocytes from healthy subjects. However, dATP and dADP concentrations are markedly higher in erythrocytes from untreated adenosine deaminase-deficient patients: 200–950 \(\mu\)mol/L (24–25) and 100–160 \(\mu\)mol/L (24, 26), respectively. dATP and dADP were not detected in this patient’s lymphocytes (see Table 1).

Previously, Chen et al. (24) reported the complete disappearance of dATP and dADP from erythrocytes from an adenosine deaminase-deficient patient after transplantation. Using a more sensitive enzymic assay, Hirschhorn et al. (25) found a substantial amount of dATP in erythrocytes from their two bone-marrow-transplant patients. Our results agree with those of Hirschhorn et al. (25), although we found that dADP was also increased in the erythrocytes from our bone-marrow-transplant patient. We have recently found an increase in adenosine and deoxyadenosine concentrations in plasma samples from this patient (21). Thus bone-marrow transplantation in adenosine deaminase deficiency did not entirely correct abnormalities in this patient’s purine metabolism.

This study has demonstrated that high-efficiency separation of purine nucleotides can be achieved with a microparticulate weak anion exchanger, and that the method is suitable for use in measuring nucleotides in human cells.

We thank P. Baltassat, for his interest in our work in the field of purine metabolism; Dr. G. Souillet, who kindly entrusted us with the biochemical study of his patient; and Mrs. N. Adla, for her secretarial assistance.

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Measurement of Plasma Fructosamine Evaluated for Monitoring Diabetes

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The mean fructosamine concentration in plasma of diabetics (n = 200) differed significantly (p < 0.001) from those of a hospitalized nondiabetic population (n = 163)—the latter mean being essentially the same for ambulatory subjects (n = 145), expectant mothers (n = 58), and patients with renal failure (n = 31), regardless of sex. In the diabetic group, values for plasma fructosamine correlated with those for glycated hemoglobin (r = 0.767, p < 0.01) and glycated protein (r = 0.817, p < 0.01). Values for plasma fructosamine were stable from day to day in patients with controlled blood glucose, but fluctuated in certain patients receiving only parenteral nutrition, even when their concentrations of glucose were normal and stable. We conclude that measurement of plasma fructosamine is suitable for assessing intermediate-term control of blood glucose when the turnover of plasma proteins is normal.

Additional Keyphrases: comparison with results for glycated hemoglobin and glycated protein • pregnancy • renal failure

A novel approach for measuring glycated proteins, based on the ability of glucose bound to protein with a ketomine linkage (fructosamine) to reduce nitro blue tetrazolium in alkaline conditions, is reportedly inexpensive and reliable for determining intermediate-term (one to three weeks) control of blood glucose (1). The measurement of plasma fructosamine has an interbatch variation of 6.5%, which compares very favorably with the intra-assay variation of 6.2% for the measurement of glycated hemoglobin (2). An advantage of measuring plasma fructosamine is that this can be automated (3, 4), thereby reducing analytical time and imprecision.

The measurement of plasma fructosamine has been used to screen for diabetes mellitus (3) and to monitor the management of non-insulin-dependent diabetes (5). We report here our findings for the normal concentrations of plasma fructosamine determined in a general hospital population, the general public (participants in a diabetic awareness project), and antenatal patients. Because the interpretation of plasma fructosamine relies upon a stable pool of vascular proteins, we also monitored several patients who were receiving only parenteral nutrition, to determine the effects of hypercatabolic states on the concentration of fructosamine in plasma.

Materials and Methods

We measured fructosamine as previously described (1), modifying the procedure for use in a CentrificChem 400 centrifugal analyzer as follows: Mix 30 μL of sample with 40 μL of distilled water, then add to 250 μL of buffered nitro blue tetrazolium reagent. Measure absorbance at 550 nm, 10 and 15 min after starting the reaction, which is performed at 37 °C. We included standards that had been previously calibrated against a 5 mmol/L standard solution of 1-deoxy-1-morpholinofructose containing 40 g of albumin per liter. For day-to-day calibration we used a control serum (QCS Normal Control Serum, lot no. 0831301; Ortho Diagnostic Systems Inc., Westwood, MA 02090) standardized

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