Isocratic Separation of ATP and Its Degradation Products from Biological Fluids by Automated Liquid Chromatography

Kim K. Tekkanat and Irving H. Fox

Two groups of metabolites (a) IMP, AMP, ADP, ATP, and cAMP in extracts of fibroblasts and erythrocytes and (b) hypoxanthine, xanthine, adenosine, and inosine in plasma and urine have been separated by ion-pairing reversed-phase chromatography on a µBondapak C18 column, with use of the following reagents: 60 mmol/L KH2PO4, 0.45 mmol/L tetrabutylammonium phosphate, and 1.26 mol/L acetonitrile, pH 3.2 (at 23 °C) (group a) and 20 mmol/L KH2PO4, 0.45 mmol/L tetrabutylammonium phosphate, and 0.35 mol/L acetonitrile, pH 2.70 (at 24 °C) (group b). Under both sets of conditions, the compounds are completely separated in <15 min. The separation is isocratic, so the method is easily adaptable to automation.

Additional Keyphrases: nucleosides · nucleotides · urine · fibroblasts · erythrocytes · reversed-phase chromatography · heritable disorders · Lesch-Nyhan syndrome · purines · economics of laboratory operation

In certain human disease states there is decreased synthesis or increased degradation of adenosine triphosphate (ATP) (1). In either situation, degradation products of ATP are released and appear in body fluids (1–5). These purine nucleosides and bases provide markers for ATP degradation in vivo. For this reason, many investigators have devised ways to measure small quantities of purines in biological samples by “high-performance” liquid chromatography (HPLC) (6–11). For best accuracy in measuring tissue purines in this way, sample-preparation and separation techniques should maximize detection capability and measurement precision while minimizing interfering matrix effects. For this to be feasible when many samples are to be processed, the time required and cost of analysis must also be considered.

In the past, nucleosides in biological samples have been resolved by anion-exclusion chromatography (12), ion-exchange HPLC utilizing an ionic gradient (13–16), or by reversed-phase liquid chromatography with use of an organic gradient modifier (17–19). Only limited information is available concerning the isocratic separation of nucleosides with these chromatographic systems (12, 17, 20). Although nucleosides and bases have been separated isocratically (21–23), hypoxanthine, xanthine, adenosine, and inosine have been most commonly separated by the use of reversed-phase HPLC with an organic gradient modifier (12, 17, 24–26). These techniques do give useful separations, but they are time consuming, because of the time required for column equilibration when ion-exchange is used and the time required for re-equilibration after gradient elution. In addition, gradient elution requires careful re-equilibration of the column between samples, to assure reproducible retention times (27). However, such close attention is not compatible with analysis of numerous samples with an automated injection system, because the instrument is often left unattended for long periods of time. A solution to this problem is to use an isocratic elution system, with similar chromatographic conditions for nucleotide and nucleoside separations.

In recent years, the use of ion-pair reversed-phase liquid chromatography for separating nucleotides and nucleosides has gained increasing attention (28–31). This technique allows the simultaneous resolution of both neutral and charged compounds on a reversed-phase column by altering the interaction of these compounds with the reversed-phase system. Under ion-pairing conditions, charged molecules form neutral ion pairs by combining with the pairing agent in the mobile phase. The neutral ion pair thus formed is retained longer in the chromatographic system, thus facilitating separation. This approach seemed to provide an ideal potential method for measuring purine nucleotide, nucleoside, and base compounds in a large number and variety of biological samples. To accomplish the task of analyzing numerous samples for ATP and its degradation products, we modified existing sample-preparation techniques and ion-pair chromatographic techniques and developed new techniques that would allow automated analysis for these compounds in the most accurate and time-efficient way.

Materials and Methods

Apparatus. A Water Associates (Milford, MA) HPLC was used in all determinations. The system consisted of a M-45 solvent-delivery system, Model 440 absorbance detector, and a Model 710B WISP (sample processor). Retention times and peak areas were recorded with a M730 data module (Waters).

Column. We used 3.9 mm × 30 cm C18 µBondapak columns with maximum efficiencies exceeding 6000 theoretical plates in all analyses. We determined column efficiency, N, by calculating the number of theoretical plates obtained for acenaphthene at 1.0 mL/min in a mobile phase of 60:40 acetonitrile:water at 4.4% of peak height, using the equation N = 1/25 (RVspec/RV4.4%) where RVspec is the retention volume at peak apex and RV4.4% is the retention volume at 4.4% of peak height (32). To protect this analytical column from particulate contamination, we used a guard column of the same C18 packing material.

Chemicals. We prepared aqueous mobile phase buffers, using potassium dihydrogen phosphate ("HPLC" grade; Fisher Scientific, Detroit, MI), tetrabutylammonium phosphate (TBAP; Eastman Kodak, Rochester, NY), and acetonitrile (Fisher) in doubly-distilled de-ionized water, which was processed to a resistance of 18 MΩ by a Milli-Q filtration system (Millipore Inc., Bedford, MA). All buffers were filtered through 0.45-µm (pore size) filters (type HA, Millipore) and de-gassed before analysis.

Standards were prepared by using bases, nucleosides, and nucleotides purchased from Sigma Chemical Co., St. Louis, MO. Stock standard solutions of 1.0 mmol/L concentrations

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were prepared in doubly-distilled water and diluted to 100, 25, and 5.0 μmol/L. The precise concentrations of these standards were determined by measuring the absorbance at λ\text{max} for each compound individually, in a Cary 210 Spectrophotometer (Varian Assoc., Palo Alto, CA). To be sure the standard compounds were pure (and to confirm that no degradation had taken place) we chromatographed each standard individually before preparing the standard mixture. This standard mixture was divided into 500-μL aliquots, which were stored at -70 °C. One such aliquot was thawed for each run, any left over being discarded.

**Chromatographic Conditions**

_Nucleoside mono-, di-, and triphosphates_ were separated with a mobile phase containing, per liter, 60 mmol of KH₂PO₄, 0.45 mmol of TBAP, and 1.26 mol of acetonitrile. The pH was adjusted to 3.20 at 23 °C with concentrated phosphoric acid. Because the pH of the mobile phase necessary for optimal separation was found to decrease slightly with increasing temperature, pH was measured at various temperatures between 19 and 24 °C and a plot was constructed. The resulting curve showed that the pH of the mobile phase decreased linearly, from 3.40 at 19 °C to 3.15 at 24 °C. From this curve the appropriate pH for optimal separation at ambient temperature could be determined.

To separate _nucleosides and bases_ we used a mixture containing, per liter, 20 mmol of KH₂PO₄, 0.45 mmol of TBAP, and 0.35 mol of acetonitrile, pH 2.70 to 3.10 (depending on temperature) as the mobile phase. We adjusted the pH with concentrated phosphoric acid. The pH of the mobile phase necessary for optimal separation of hypoxanthine, xanthine, adenosine, and inosine was determined at several temperatures between 19 and 24 °C, and a curve of pH vs temperature was constructed. Here, pH was found to decrease linearly with increasing temperature. From this curve, the pH of the mobile phase necessary for optimal separation of these compounds at ambient temperature could be ascertained.

**Sample Preparation**

In all cases, samples were prepared so as to maximize analytical recovery of the components of interest and at the same time minimize interferences from other ultraviolet-absorbing compounds.

_Cultured fibroblast extracts._ Human fibroblast monolayers were prepared for nucleotide analysis by first discarding the medium and gently washing the monolayer two to three times with Hank’s balanced salt solution (Gibco, Chagrin Falls, OH). Nucleotides were then collected in 1.5 mL of 0.60 mol/L ice-cold trichloroacetic acid (Fisher Scientific) and neutralized with 1.5 mL of a 50 mmol/L solution of tri-n-octylamine (Sigma) in Freon 113 (J.T. Baker Chemical Co., Phillipsburg, NJ) according to the method described by Khyr (33). The neutralized extracts were filtered through 0.45-μm (pore size) nitrocellulose filters (Anspec, Ann Arbor, MI) before analysis. We determined analytical recoveries by using [8-¹⁴C]nucleotide compounds from ICN, Irvine, CA.

_Erythrocyte extracts._ Human erythrocyte extracts were prepared for analysis by adding an equal volume of whole blood to 0.73 mol/L trichloroacetic acid (on ice), vortex-mixing, and centrifuging to separate the supernate from the cells. The supernatant fluid was then removed from the pellet and added to an equal volume of the tri-n-octylamine/Freon 113 mixture, vortex-mixed for 2 min, and centrifuged (1700 × g, 5 min) in a refrigerated centrifuge. The aqueous (top) layer was filtered through an Anspec nitrocellulose filter before analysis.

_Plasma._ Blood samples from human subjects were collected into heparinized tubes (on ice) containing, per liter, 10 μmol each of dipyridamole and erythro-9-(2-hydroxy-3-nonyl)adenine (both from Burroughs Wellcome, Research Triangle Park, NC) and centrifuged without delay (1900 × g, 10 min, 4 °C). The plasma supernates were removed from the cells and stored at -20 °C until further processing.

Before analysis, we divided each plasma supernate into a 0.5-mL control sample and a 0.5-mL "enzyme" sample. Enzymes were diluted in phosphate buffer (30 mmol/L, pH 7.4) to give the desired concentrations. We added 80 μU of uricase (EC 1.7.3.3; U-3250, Sigma) to the control sample, and then incubated for 1 h at 37 °C to remove uric acid; otherwise, uric acid interfered by co-elution with hypoxanthine under the given chromatographic conditions. (We had first tested the uricase with a standard compound of hypoxanthine, xanthine, adenosine, and inosine to ensure that no contaminating enzymes were present in the uricase to degrade these compounds.) To the 0.5-mL "enzyme" sample we added adenosine deaminase (EC 3.5.4.4; 0.94 U, cat. no. A-1030), xanthine oxidase (EC 1.1.3.22; 0.23 U, X-4500), purine-nucleoside phosphorylase (EC 2.4.2.1; 0.65 U, N-3003), and uricase (0.038 U, U-3250), all from Sigma Chemical Co., then incubated for 1 h at 37 °C to degrade plasma purines.

To optimize conditions for sample preparation, we compared three different methods of deproteination:

1. Membrane cone ultrafiltration. We centrifuged 0.5–1.0 mL of plasma in Type CF-25 Centrifflo membrane cones (Amicon Corp., Danvers, MA) at 1700 × g for 30 min, then re-filtered the filtrate through a 0.45-μm (pore size) filter (Anspec) before analysis by HPLC.

2. Pre-column cartridge. Plasma samples could also be deproteinized via Sep-Pak C₁₈ cartridges (Waters Associates) under conditions whereby the nucleosides and bases were retained on the column and then selectively eluted. In this method, we pre-wet the C₁₈ cartridge with 2 mL of an equilum-solution of methanol and water, then equilibrated (washed) the column with 10 mL of 50 mmol/L KH₂PO₄ buffer (pH 4.5). We next loaded 0.5 mL of plasma onto the Sep-Pak cartridge, followed by 1 mL of the phosphate buffer to remove weakly- to moderately-retained compounds. We then eluted the nucleosides and bases with 2 mL of an equilum mixture of tetrahydrofuran (J.T. Baker) and water, then evaporated the tetrahydrofuran, leaving the compounds of interest in the aqueous phase. Before HPLC analysis, we filtered the samples through 0.45-μm filters.

3. Micropartition filtration system. We also investigated using Amicon’s MFS-1 filtration system. With this method we could deproteinize and filter the sample in one step by placing the filter (Millipore, Type HA) underneath the deproteinization membrane, placing plasma in the filtering device, and centrifuging at 1700 × g for 15 min.

_Urine._ Urine samples were collected into 1 mL of toluene (to prevent bacterial growth) and stored at -20 °C until analyzed. Before analysis, we diluted the samples 10-fold in doubly-distilled water and divided each into two 0.5-mL aliquots. As with the plasma samples, we added uricase to the control sample and xanthine oxidase, nucleoside phosphorylase, and uricase to the "enzyme" aliquot, to degrade urinary purines. Because of the higher concentrations of purines in urine, we used twice as much enzymes as for the
plasma samples. We incubated the samples for 2 h at 37 °C and then proceeded with one of the plasma-deproteinization methods described above.

Identification and Quantification of Compounds

Nucleotides, nucleosides, and bases were identified by comparing their retention times with those for standard compounds. To further corroborate the identities of nucleotides found in erythrocyte and fibroblast samples, we collected the peak fractions, hydrolyzed the pyrophosphate bond, and identified the corresponding nucleoside or base with the chromatographic conditions described for nucleosides. Identities of hypoxanthine, xanthine, and inosine in plasma and urine and adenosine (plasma only) were further confirmed via enzymatic removal of peaks (2). If some ambiguity as to peak composition remained after all these procedures, we further confirmed peak purity by co-chromatographing standards and samples, and we compared sample absorbance ratios (280 nm/254 nm) with those for standard compounds. We made an ultraviolet spectral scan of the column effluent corresponding to the collected peak when further confirmation of peak identity was required.

The enzymatic peak-removal technique we used is a variation of the enzymatic peak-shift technique (34), which involves incubating a sample with a specific enzyme, such that the peak for the enzyme substrate disappears and a new peak (corresponding to the product) appears in a different region of the chromatogram. In the current technique, we added enzymes to degrade all of the purine nucleosides and bases of interest (i.e., hypoxanthine, xanthine, adenosine, and inosine) to uric acid. We also added uricase to remove uric acid (the end product of this pathway in humans). Consequently, all purine peaks disappeared and no new peaks appeared. To assure complete degradation of purines under the enzyme incubation conditions, we tested each new enzyme mixture by adding it to a standard mixture of hypoxanthine, xanthine, adenosine, and inosine and subsequently monitoring this for complete peak removal.

After the peaks were identified unambiguously, we determined the concentrations of each compound on the basis of peak area via the use of external standards. In this way, we obtained sample concentrations by calibrating with a standard solution having known concentrations. We then calculated response factors (RF = C/A, where C is the standard concentration and A is the peak area produced by that concentration) for each analyte and multiplied this by the sample area and an appropriate scale factor (i.e., the dilution factor) to obtain concentrations of the analytes in the samples.

During optimization of separation conditions, we calculated capacity factors (k') for each compound in standard mixtures, relating the retention volume or retention time of the retained compound, t_r, with that of a compound that is not retained on the column, t_0, by the expression: k' = (t_r - t_0)/t_0.

Results

Sample-Preparation Techniques

Nucleotides. For fibroblast extracts deproteinized with the strong acid (trichloroacetic acid) neutralization technique described by Khym (33), mean (± SD) analytical recoveries of (8-14C)-labeled compounds were: AMP, 89% ± 2%; IMP, 95% ± 1%; ADP, 93% ± 2%; and ATP, 98% ± 1%. Considerably less cAMP was accounted for, 65% ± 8% (n = 10). Recoveries from erythrocyte extracts were lower than those for fibroblast extracts: AMP, 79% ± 6%; IMP, 82% ± 3%; ADP, 82% ± 5%; and ATP, 85% ± 1% (n = 8). Recovery of cAMP was not assessed. All (100%) of the labeled nucleotides were accounted for in aqueous standards of nucleotide compounds extracted by this procedure.

Nucleosides and bases. The methods of sample preparation were examined with respect to protein removal, recovery of nucleosides and bases, deletion of interfering ultraviolet-absorbing compounds, and ease and speed of use. All of the methods produced good recoveries with standard compounds, but recoveries of purines in plasma and urine varied with the method used (Table 1). To determine analytical recovery in plasma samples, we added each purine to the sample to give a 5 µmol/L final concentration of each and measured how much of that could be accounted for after the deproteinization step. Recoveries of purines from the samples after deproteinization were measured by essentially the same method, except that we added 10 µmol/L concentrations of purines to the urine samples, which had been diluted 10-fold.

Of course, there are advantages and disadvantages associated with each of the deproteinization techniques described above. With both the cone-ultrafiltration and Sep-Pak methods, deproteinization was quite efficient, only 50 to 100 mg of protein per liter being measurable after deproteinization of a 500-µL plasma sample, as assessed in 20 randomly selected plasma samples prepared by each method and measured for protein by the Bradford method (35). The MPS-1 filtering system was even more efficient, <25 mg/L being detectable after filtration through new filters. Al-

Table 1. Comparison of Purine Recoveries after Different Deproteinization Techniques

<table>
<thead>
<tr>
<th>Compound</th>
<th>Plasma %</th>
<th>Standard %</th>
<th>Urine %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cone</td>
<td>Sep-Pak</td>
<td>MPS-1</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>98 ± 2</td>
<td>96 ± 1</td>
<td>96 ± 3</td>
</tr>
<tr>
<td>Xanthine</td>
<td>97 ± 2</td>
<td>98 ± 2</td>
<td>98 ± 1</td>
</tr>
<tr>
<td>Adenosine</td>
<td>80 ± 5</td>
<td>88 ± 1</td>
<td>86 ± 2</td>
</tr>
<tr>
<td>Inosine</td>
<td>83 ± 4</td>
<td>96 ± 3</td>
<td>95 ± 4</td>
</tr>
</tbody>
</table>

*All values represent the mean (± SD) percent accounted for—i.e., analytical recovery.

**6 µmol/L standard mixture (n = 15).

† To each plasma sample we added 5 µmol of each purine per liter and determined how much could be accounted for after the deproteinization step (n = 10).

‡ We added 10 µmol of hypoxanthine, xanthine, and inosine per liter to the 10-fold diluted urine and determined the percent accounted for after deproteinization (n = 10).

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though the manufacturer does not recommend it, we soaked the filters for 2 h in 0.1 mol/L NaOH, rinsed several times in doubly-distilled water, and re-used them. After 10 uses of the filter, the mean concentration of protein remaining in 15 different prepared plasma samples was 84 (SD 12) mg/L. To determine whether contaminating compounds were present on the filter after the soaking process, we passed a sample of water through a pre-soaked filter that had been used five time previously and chromatographed the filtrate; no contaminating compounds were detected.

As for overall purine recovery and ease of use, both filtering methods perform better than the Sep-Pak precolumn cartridge deproteinization method. However, the Sep-Pak technique, although time consuming, provides a means of obtaining "cleaner" samples because compounds can be removed by selective elution. We found this technique to be most advantageous when dealing with plasma samples from critically ill patients or from dialysis patients because many of the ultraviolet-absorbing compounds that interfere with the detection of purine peaks could be removed. However, if values with this method are to be accurate, one must take extreme care not to dilute the sample.

The cone ultrafiltration method for protein removal was easier to use than the Sep-Pak method, and it yielded good recoveries of purines from plasma and urine without introducing errors from dilution as long as the cones were pre-centrifuged as prescribed by the manufacturer. Moreover, because the cones could be re-used many times, they provided a cost-efficient means of sample preparation. The cone ultrafiltration technique was the best of the three when the plasma samples contained large amounts of protein. The only disadvantage was that analytical recovery of adenosine was lower than that for the other purines.

Overall, we found MPS-1 filtration to be the best method of deproteinization for routine use, yielding good recovery of all purine compounds studied and being the easiest to use. As an added advantage, protein removal and particulate filtration could be accomplished in one step; and, because the MPS-1 filters are re-usable, the method was also cost efficient.

**Analytical Variables**

The lower limit of detectability for all the compounds we investigated was 0.1 μmol/L (ultraviolet detector sensitivity = 0.005 absorbance unit full scale, 50-μL injection volume). This limit was determined with a standard mixture containing 0.1 μmol (as determined with ultraviolet spectroscopy) each of AMP, IMP, ADP, and ATP per liter. The mean concentration determined by HPLC for these compounds was 0.1 μmol/L with standard deviations 0.01, 0.01, 0.03, and 0.04, respectively (n = 10). The lower limit of detectability for hypoxanthine, xanthine, adenosine, and inosine was determined similarly with a 0.1 μmol/L standard mixture of each compound. The mean measured concentration for all compounds was 0.1 μmol/L, with standard deviations 0.01, 0.01, 0.02, and 0.04, respectively (n = 10). Absorbances varied linearly with concentration from 0.1 to 100 μmol/L for each nucleotide, nucleoside, and base in the standard compounds. Run-to-run precision was assured by comparing areas of standard peaks from identical injection volumes of the same pre-prepared and stored (-70 °C) standards (Table 2). Both plasma and urine are complex biological matrices, so between-run precision was confirmed by analyzing a control sample with every run; coefficients of variation for nucleosides and bases in these samples are also shown in Table 2.

**Chromatographic Separations**

**Nucleotide separation.** The chromatographic conditions we used to separate nucleotides in human fibroblast and erythrocyte extracts were adapted from those of Darwish and Prichard (31). We modified the mobile phase to contain, per liter, 60 mmol of KH₂PO₄ (pH 3.2 at 23 °C), 0.45 mmol of TBAP, and 1.26 mol of acetonitrile. Given this proportion of acetonitrile and the use of a C₁₈ stainless-steel column, we could decrease the flow rates from 2.8 mL/min (31) to 1.8 mL/min, which resulted in more efficient separation (primarily determined by the number of theoretical plates) (32). Complete separation of AMP, IMP, ADP, and ATP (and cAMP, when detectable) could be attained isocratically in <15 min (Figures 1–3). Mean (and SD) values, in millimoles per liter, obtained for normal erythrocyte nucleotides (nine samples each from five healthy men) chromatographed under these conditions were: ATP, 1.21 (0.19); ADP, 0.58 (0.02); AMP, 0.16 (0.02); and IMP, 0.08 (0.01). Mean (and SD) nucleotide concentrations, in nanomoles per milligram of protein, in normal cultured fibroblasts (six different cultures) were: ATP, 35.8 (2.3); ADP, 2.82 (0.27); AMP, 0.97 (0.12); and IMP, 0.44 (0.05).

![Fig. 1. Chromatogram for a 50-μL injection of a standard compound of AMP, IMP, ADP, ATP, and cAMP (2 nmol/50 μL each) onto a C₁₈ µBondapak column eluted with mobile phase A (pH 3.20 at 23 °C) at a flow rate of 2.0 mL/min. Sensitivity, 5 milli-absorbance unit full scale](image-url)
In an attempt to shorten run time while maintaining optimal resolution, we examined several different variables affecting the separation. We determined that the concentration of the pairing agent (TBAP) could be varied from 0.45 to 0.90 mmol/L without noticeable change in the separation, and that the size of the cation in the mobile-phase buffer, for example, K⁺[(H₂PO₄)]⁻ vs NH₄⁺[(H₂PO₄)]⁻, was not an important variable. Ionic strength, concentration of acetonitrile, overall efficiency of the column, and the pH of the mobile phase did affect the nucleotide separation. Separation was optimal when the ionic strength of the potassium dihydrogen phosphate buffer in the mobile phase was 60 to 70 mmol/L. At phosphate concentrations <50 mmol/L, the retention time of ATP was too long and its corresponding peak too broad for accurate quantification of low concentrations. Similarly, if the concentration of acetonitrile in the mobile phase was <1.26 mol/L, late-eluting compounds were more difficult to quantify. If the acetonitrile concentration was >1.3 mol/L, retention times were decreased at the expense of resolution. With use of 60 mmol/L phosphate buffer and 1.26 mol/L acetonitrile, even the latest-eluting peak was easily detectable and quantifiable. Even so, all compounds were well resolved only when the overall efficiency of the column exceeded 4500 theoretical plates.

Relatively small changes in pH altered the capacity factor, k', for each nucleotide. At pH 3.30 (24 °C) the capacity factors for AMP and IMP are the same and the two compounds are co-eluted. At lower pH—e.g., between pH 3.00 and 3.15 at 24 °C—there is complete separation of AMP, IMP, and ADP. Above pH 3.35 the nucleotides begin to separate into distinct peaks again, but now IMP is eluted before AMP; all compounds are again completely resolved above pH 3.75. When the pH of the nucleotide mobile phase buffer exceeds 3.30, the capacity factor for each compound increases as the pH increases. Although all compounds can be completely resolved, ATP is usually eluted much later and hence is difficult to quantify in low concentrations.

Nucleoside separation. Conditions used to separate nucleosides and bases in plasma and urine are similar to those described above for the nucleotide separations, except for the mobile phase, which contained, per liter, 20 mmol of KH₂PO₄, 0.45 mmol of TBAP, and 0.35 mol of acetonitrile, pH 2.75. Hypoxanthine, xanthine, adenosine, and inosine could be completely separated within 12 min under these conditions (Figures 4 and 5). Although pH does not induce a large change in retention behavior for hypoxanthine, xanthine, or inosine, small variations in pH at constant temperature result in large changes in the capacity factor for adenosine. Separation at 24 °C is best when the pH of the mobile phase is between 2.7 and 2.8.
Fig. 5. Chromatogram for a 40-μL injection of 10-fold-diluted urine from a normal subject receiving a fructose infusion.
Flow rate, 1.5 mL/min; abbreviations and other chromatographic conditions as in Fig. 4.

Table 3 gives values for plasma nucleosides and bases in normal subjects, in samples prepared with the MPS-1 filtration system and analyzed under these conditions. Values for purines in plasma from critically ill patients were higher and more variable. Data for such samples (prepared in the same way as samples from normal subjects) are also shown in Table 3.

Normal values for purines in urine, obtained with the use of the MPS-1 deproteinization system and analyzed under the same conditions, are shown in Table 3. These values for patients with metabolic disorders frequently are much higher than normal. For example, a patient with Lesch-Nyhan syndrome, a disease that involves deficiency of hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8), excreted >10-fold more purines in the urine than did normal subjects (Table 3).

Although plasma and urine samples were analyzed only for hypoxanthine, xanthine, inosine, and adenosine (in plasma only), our system can also be used to separate a range of other nucleosides and bases (Figure 6). The ability to separate these compounds was useful in confirming the identity of nucleotide peaks through the use of a phosphate hydrolysis reaction of the peak fraction and the subsequent analysis of the nucleoside product formed.

Discussion

Ion-pair reversed-phase HPLC can be used effectively to separate and analyze for ATP and its degradation products in a large number of biological samples if automated sample injection is used. Use of ion-pair chromatography to separate these specific nucleotides was much less time consuming than ion-exchange HPLC (13-16), and its elution characteristics were superior to those of reversed-phase HPLC (17); with the latter technique, ADP is not always completely separated from ATP. In addition, because AMP was eluted early in the ion-pairing chromatographic system instead of being the last compound to elute in the reversed-phase system, it was more easily and accurately quantified.

Although others have separated standard compounds of nucleosides by ion-pair chromatography (28), the technique has not yet been used to separate hypoxanthine, xanthine, adenosine, and inosine isocratically in plasma and urine. We found this chromatographic technique to be very suitable for the rapid measurement of these compounds under automated conditions. Probably the major advantage of this ion-pair system is that purine compounds are easily detectable and quantifiable in urine samples and plasma samples from critically ill patients prepared for HPLC by standard methods. This ease of sample preparation contrasts with the elaborate off-line chromatographic techniques that have been used previously to prepare urine samples for the reversed-phase HPLC detection and quantification of purines (36, 37). Such extensive preparation had been necessary for quantifying purines, because other ultraviolet-absorbing compounds in the urine, eluting at the same retention times, obscured the purine peaks.

The HPLC procedures for purine analysis were time efficient, and were highly accurate and reproducible. In our studies, analytical recovery for nucleotides in erythrocyte samples prepared according to the methods described by

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Table 3. Values for Purines in Plasma and Urine

<table>
<thead>
<tr>
<th>Sample</th>
<th>Hyp (μmol/L)</th>
<th>Xan (μmol/L)</th>
<th>Ado (μmol/L)</th>
<th>Ino (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal b</td>
<td>1.0 ± 0.2</td>
<td>0.7 ± 0.2</td>
<td>0.4 ± 0.1</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>Critically ill c patients</td>
<td>6.4 ± 2.5</td>
<td>3.7 ± 0.7</td>
<td>0.9 ± 0.4</td>
<td>0.6 ± 0.4</td>
</tr>
<tr>
<td>Urine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal d</td>
<td>33.4 ± 8.8</td>
<td>24.0 ± 15.8</td>
<td>12.2 ± 6.4</td>
<td></td>
</tr>
<tr>
<td>Lesch-Nyhan e</td>
<td>8182 ± 5121</td>
<td>4283 ± 3227</td>
<td>746 ± 456</td>
<td></td>
</tr>
</tbody>
</table>

*All values represent mean ± SD; all samples were deproteinized with the MPS-1 filtration system.

b Data represent results obtained from analysis of nine samples each from five healthy men.

c Two samples were analyzed from each of five different patients.

d Same as in b.

e Value for six 24-h urine samples from a single patient.

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Fig. 6. Chromatogram for a 40-μL injection of a standard mixture of nucleosides and bases.
Cyd, cytidine; Cyt, cytosine; Ade, adenosine; Guo, guanosine; Ino, inosine; dThd, thymidine; Xao, xanthosine. Chromatographic conditions and other abbreviations as in Fig. 4.
Khym (33) were similar to or better than those obtained by previous investigators (38). Normal values for di- and tri-
nucleotides fell within the ranges of values obtained by
other investigators [e.g., ATP 1.0–3.5 mmol/L, ADP 0.15–
0.64 mmol/L (39–42)], but concentrations of IMP and AMP
in normal erythrocytes were higher than those previously
reported [AMP, 10–62 μmol/L; IMP 0–14 μmol/L (39–42)].
These discrepancies may reflect differences in recovery of
the monophosphate nucleosides during sample preparation.
Our sample-preparation procedures for nucleosides and
bases also resulted in recoveries comparable with those
previously reported, but recoveries depended greatly on the
associated matrix such as plasma or urine (37). Although
the Sep-Pak method was useful for removing other ultraviolet-absorbing compounds and the ultrafiltration membrane
cones may be more durable, we used the MPS-1 filtration
system routinely for protein removal because it offered
the overall best recovery and was easiest to use. Values for
purines in normal plasma were within the range of values
described by previous investigators: hypoxanthine, 370–
1940 nmol/L (22, 43–45); xanthine, 370–890 nmol/L (22, 44,
45); adenosine, 40–410 nmol/L (22, 44, 46); and inosine, 80–
1620 nmol/L (6, 43–46). Values for urinary hypoxanthine
and xanthine were in the range of values reported by Kurzt
et al. (45), which were: hypoxanthine, 46.3 (SD 18.7) nmol/
mg of creatinine; and xanthine, 35.9 (SD 16.2) nmol/mg of
creatine. Moreover, we could detect urinary inosine,
whereas others could not (45).

Although ion-pair chromatography appears to be very
useful for determining adenine nucleotide and nucleoside
compounds in various biological matrices, careful control of
chromatographic conditions is required if results are to be
reproducible.

Factors most affecting nucleotide separation include ionic
strength, acetonitrile concentration, and the pH of the
mobile phase; and the efficiency of the C18 column. Resolution is optimal when the acetonitrile concentration in the
mobile phase is 1.35 mol/L, the pH of the mobile phase is in
the range between 3.15 and 3.20 (inclusive), and the overall
efficiency of the column exceeds 5000 theoretical plates at a
flow rate of 2.0 mL/min.

Factors most affecting nucleoside separation include pH,
temperature, and efficiency of the column. Under the given
ion-pairing conditions, the elution order of the nucleosides
and bases is what one might predict, based on their dissociation constants: hypoxanthine, 8.8; xanthine, 7.53; adeno-
sine, 3.6; and inosine, 1.1 (47, 49). Although hypoxanthine,
xanthine, and inosine are virtually unaffected by small (0.1
unit) pH changes, the retention behavior of adenosine is
greatly affected. When the pH of the mobile phase is
decreased below pH 3.6, the purine ring nitrogen of adeno-
sine is protonated, which causes the compound to have less
affinity for the hydrophobic C18 stationary phase and hence
to elute earlier. As the pH of the mobile phase is increased
above 3.6, the base loses this charge and k' increases
dramatically. Because these small fluctuations in mobile-
phase pH are greatly influenced by temperature, either the
effects of temperature must be compensated or the temperature at which the separation takes place should be con-
trolled. We chose to determine the effects of temperature
on the mobile phase and adjusted the pH accordingly, with very
reproducible results. One could also simply control the
column temperature by using a thermostated column jacket.
Indeed, that may be a more reliable method for controlling
the separation, and it should be used when available. Lastly,
overall column efficiency must be greater than 5000 theoretical
plates for optimal resolution of all compounds.

The use of ion-pair chromatography has two major advan-
tages over ion-exchange chromatography or reversed-phase
chromatography. First, the C18 column is very durable if a
guard column is used and the C18 column is properly and
routinely cleaned before storage. In addition, unlike ion-
exchange columns, C18 columns do not require long equili-
bration times; therefore, more time can be devoted to sample
analysis. The number of samples analyzed was further
increased through the elimination of column change-over
time, because the same column was used in all analyses.
Second, and most importantly, the use of a pairing agent
improved the detection limits of purines in complex matrices,
because other components in the matrix were converted
into nonultraviolet-absorbing ionic compounds. Thus in a
clinical research setting, ion-pair chromatography would be
especially useful when analyzing plasma samples from
critically ill patients or concentrated urine samples, which
often contain many ultraviolet-absorbing compounds. The
use of ion-pair chromatography with the sample-preparation
procedures described will facilitate studies aimed at
examining ATP and its degradation products.

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