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Note

Simultaneous analysis of ATP, ADP, AMP, and other purines in human erythrocytes by high-performance liquid chromatography*

PAUL D. SCHWEINSBERG** and TI LI LOO

Department of Developmental Therapeutics, The University of Texas System Cancer Center M.D. Anderson Hospital and Tumor Institute, Houston, Texas 77030 (U.S.A.)

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The nucleotide profile of human erythrocytes is very simple when compared with nucleated mammalian cells. Having little or no pyrimidines, the human erythrocyte nucleotide profile consists exclusively of purines. Since DNA is not synthesized, only ribonucleotides are detected. The adenine ribonucleotide pool, consisting of ATP, ADP, and AMP, is both abundant and important for erythrocyte function and survival. The concentration of ATP is normally 1200—1700 μmoles/ml packed erythrocytes [1—3]. ADP and AMP concentrations are generally 5—8 and 50—100 times less than ATP concentrations, respectively. The guanine ribonucleotide pool (GTP, GDP, and GMP) as well as other purine pools, are much smaller than the adenine ribonucleotide pool in metabolically normal erythrocytes.

In our studies of metabolism and mechanism of action of antitumor agents, we found it desirable to monitor erythrocyte pools of purine nucleotides, nucleosides, and bases simultaneously. Purine nucleotides, but not nucleosides or bases, are resolved well on strong anion-exchange columns [4—6], and therefore a reversed-phase technique was developed which gives good resolution of ATP, ADP, and AMP, as well as partial resolution of other purine constituents. The ionic strength, pH, and gradient program of the mobile phase buffers were optimized for maximum resolution and precision, and minimum analysis time. Samples can be analyzed every 30 min, with relatively little solvent used.

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** To whom correspondence should be addressed.

EXPERIMENTAL

Instrumentation

A Waters Model ALC-204 liquid chromatograph equipped with two Model 6000A pumps, a U6K injector, and a Model 660 solvent programmer, all of Waters Assoc. (Milford, Mass., U.S.A.) was used for all analyses.

Separations were achieved on a reversed-phase μBondapak C_{18} column (4 mm × 30 cm) also from Waters Assoc.

The detector was a Varian Vari-Chrom (Varian Assoc., Palo Alto, Calif., U.S.A.). Peaks were electronically integrated with a Varian CDS-111 integrator and printed out on a Varian Model 9176 recorder.

Materials

All purine standards and crystalline potassium dihydrogen phosphate were purchased from Sigma (St. Louis, Mo., U.S.A.). Potassium monohydrogen phosphate was purchased from J.T. Baker (Phillipsburg, N.J., U.S.A.). Glass distilled methanol was obtained from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.). Blood was obtained by venipuncture of healthy volunteers.

Buffer preparation

Buffers are diluted from a 1.0 *M* stock solution of 0.6 *M* K_2HPO_4 and 0.4 *M* KH_2PO_4.

Buffer A. Buffer A is prepared by diluting 100 ml of the stock to 1 l with distilled water. The pH is adjusted to 6.0 with concentrated phosphoric acid, and the solution is filtered through a Millipore 0.4-μm filter (Millipore, Bedford, Mass., U.S.A.).

Buffer B. Buffer B is prepared by diluting 100 ml of the stock solution to 750 ml with distilled water. The pH is adjusted to 6.0 and filtered as described above. To the filtrate is added 250 ml glass distilled methanol with constant stirring. The buffer is stirred for several hours at room temperature before use.

Standard solutions

All 17 purine standards are individually dissolved in appropriate solvents to a concentration of 3 m*M*. A mixture is obtained by combining an equal volume of each.

Sample preparation

Aliquots of thoroughly washed erythrocytes are packed by centrifugation at 12,000 *g* for 10 min at 4° on a Sorvall RC2-B refrigerated centrifuge. The packed cells are extracted for 30 min on ice with 2.5 volumes of 0.56 *N* perchloric acid (PCA). The insoluble material is removed by centrifugation, and the supernate is collected and neutralized with 10 *N* KOH. The $KClO_4$ is discarded after centrifugation and the resulting supernate is analyzed directly. Concentrations of purine constituents are determined by the peak area per nmole relationship of known concentrations of purine standards.

Chromatographic conditions

System I. For complete analysis of purine pools, a 30-min concave gradient

(curve 10 on the solvent programmer) from 0% Buffer B to 100% Buffer B is run at 1 ml/min. The eluent is monitored at 254 nm, with full scale deflection set at 0.2 absorbance units when 50-μl aliquots of the erythrocyte extracts are applied. After 40 min the column is re-equilibrated in 10 min by immediate reversal to 100% Buffer A.

System II. For a more rapid analysis of adenine ribonucleotide pools, Buffer A is pumped isocratically for 10 min at 1 ml/min followed by isocratic application of Buffer B for 10 min at 1 ml/min. The column is then re-equilibrated as above in 10 min.

RESULTS

Fig. 1 shows the separation of 17 purine standards using System I. Fig. 2 shows the separation of the purine standards using System II. With either system, the retention times of all compounds that are eluted in the first 15 min

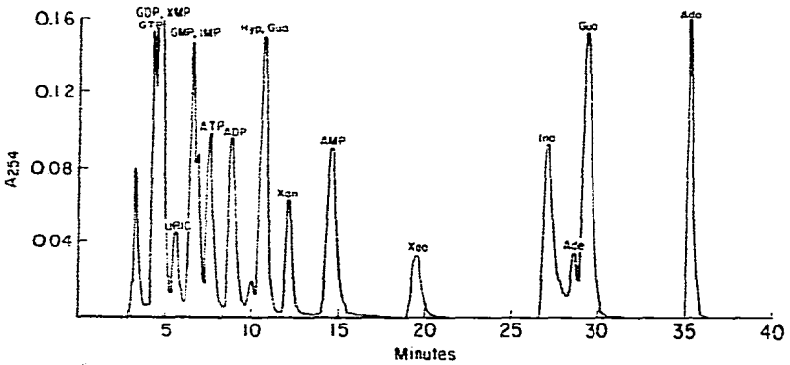

Fig. 1. Separation of approximately 3.5 nmole each of 17 purine standards using System I (20 μl injected).

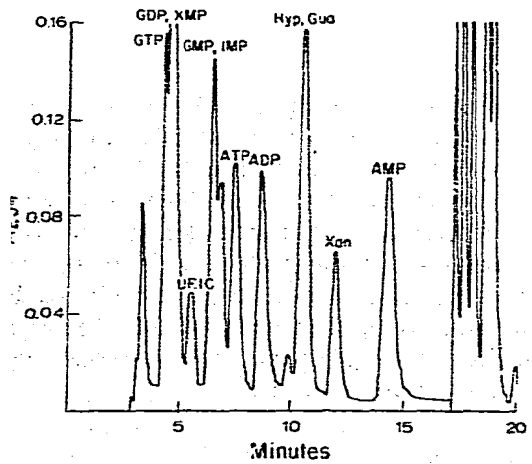

Fig. 2. Separation of approximately 3.5 nmole each of 17 purine standards using System II (20 μl injected).

are identical. Poor resolution of the later eluting compounds occurs with System II. Several compounds, including the guanine ribonucleotides, co-elute with other purine standards as experimental conditions are optimized for analysis of adenine ribonucleotides and common nucleosides such as inosine and adenosine.

A typical nucleotide profile of erythrocytes from a fresh specimen of venous blood drawn from a healthy subject is seen in Fig. 3. System II is used for this chromatogram, since little if any of the later eluting compounds are found in metabolically normal erythrocytes. ATP, ADP, and AMP levels are approximately 1440, 225, and 35 μmoles/ml of packed cells, respectively.

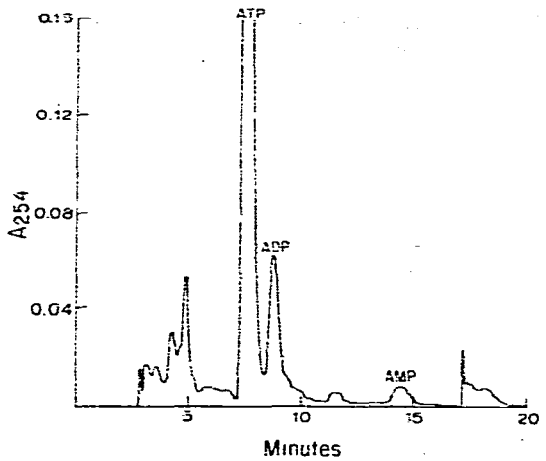

Fig. 3. Fractionation of 50 μl of PCA-soluble material obtained from human erythrocytes using System II.

DISCUSSION

The high-performance liquid chromatography technique described in this paper has several advantages. The relatively slow flow-rate enables multiple analysis without solvent change. Although two buffers are required, virtually no baseline drift occurs and almost all UV-absorbing compounds are removed from the column following each run. PCA extracts of erythrocytes can be routinely monitored at 0.2 a.u.f.s., which appreciably reduces electronic noise. Although several of the purine standards co-elute, none co-elute with ATP, ADP, or AMP, the nucleotides of interest. The method is fast and reliable for estimating adenine ribonucleotide concentrations in erythrocytes, and could be applied to many enzyme assays involving purine constituents.

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