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SELECTIVE ANALYSIS FOR ADENOSINE USING REVERSED-PHASE HIGH-PRESSURE LIQUID CHROMATOGRAPHY

RICHARD A. HARTWICK and PHYLLIS R. BROWN

Department of Chemistry, University of Rhode Island, Kingston, R.I. 02881 (U.S.A.)

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SUMMARY

A high-pressure liquid chromatographic procedure for the selective determination of adenosine in the presence of other nucleic acid components is reported. Reversed-phase microparticle columns and an isocratic elution mode of dilute potassium dihydrophosphate and anhydrous methanol were used. The analysis is specific for adenosine and is achieved in less than 10 min. An example of the use of this analysis in a biomedical study is reported.

INTRODUCTION

The analysis of nucleotides in blood is now being carried out routinely by high-pressure liquid chromatography (HPLC) [1—7], and work is in progress to determine alterations caused by various disease states of free nucleotide concentrations in physiological fluids and cell extracts [8—11]. Less attention has been paid to nucleoside levels in cells. However, recent investigations have focused attention on nucleosides in cells [12,13,39,40], and especially on adenosine concentrations which have been found to be important in cardiac disease and certain birth defects. Adenosine is one of the physiological regulators of coronary blood flow and it has been observed that there is an increase in intracellular adenosine levels in cardiac hypertrophy and after brief periods of myocardial ischemia and hypoxia [14, 15]. It has also been found that excess adenosine in cultured mammalian cells is toxic [16] and it has been postulated that severe combined immunological defects in children, in some instances, are caused by an accumulation of adenosine which is a result of an adenosine deaminase deficiency [17]. In addition, although adenosine has potential for use as a preservative in blood storage [18] and in the treatment of the Lesch Nyhan syndrome, its use has been limited because of possible toxic effects.

Research on problems involving adenosine has been hampered by the lack of an adequate analytical method which permits the sensitive and quantitative analysis of adenosine in the presence of other UV-absorbing compounds present in cellular extracts. Therefore, the objective of this research was to develop

a rapid procedure for the selective determination of adenosine. Since HPLC is uniquely suited for nucleotide and nucleoside analyses [19—37] various modes of this technique were investigated for use in an adenosine assay. It was found that the reversed-phase partition mode provided the required efficiency and selectivity using totally porous, chemically bonded microparticle packings.

EXPERIMENTAL

Chemicals

The potassium dihydrophosphate was from Mallinckrodt (St. Louis, Mo., U.S.A.), reagent-grade quality, and was used without further purification. The methanol was reagent-grade, anhydrous. The nucleoside standards: Cytidine (Cyd), Uridine (Urd), Thymidine (dThyd), Adenosine (Ado), Guanosine (Guo), Inosine (Ino) and Xanosine (Xao), their nucleotides and their bases, were from Sigma (St. Louis, Mo., U.S.A.).

Apparatus

A liquid chromatograph (Waters Assoc., Milford, Mass., U.S.A.), Model ALC 202 with a double-beam micro-UV-detector operating at a fixed wavelength of 254 nm was used. The instrument was equipped with dual pumps and solvent programmer. For the Ado analysis however, only a single M6000 pump was used in the isocratic mode. The column (30 cm × 4 mm) was packed with μBondapak C_{18} (Waters Assoc.). Integration was done electronically using a Hewlett-Packard Model 3380A integrator.

Procedures

The distilled water used in preparing the eluents was first filtered through a Millipore filter, type HA (pore size, 0.45 μm). The pH of the buffer solutions was adjusted with H_3PO_4 to 5.8, prior to the addition of methanol. In order to dissolve the nucleosides, the standards were made up in 0.007 *F* KH_2PO_4 (pH 7.8). After the standards were dissolved, the pH was adjusted to 5.8 and the solution brought up to volume.

Operating conditions

The operating conditions were optimized to obtain a selective analysis for Ado in the presence of other naturally occurring nucleosides, nucleotides and their bases. The eluent used was a solution of anhydrous methanol—0.007 *F* KH_2PO_4, pH 5.8 (10:90 v/v). The temperature was ambient. The flow-rate was 2.0 ml/min, which produced a pressure drop of approximately 1500 p.s.i. Injections of the samples were via the Waters' U6K injector (a modified sample-loop type of injector). Hamilton syringes (10, 25 or 100 μl) were used to inject the sample volumes.

Cell extraction procedures

The extraction procedure used for the blood samples is that described by Khym [38]. In this procedure, 1 ml of the sample was denatured by the addition of 2 ml of cold trichloroacetic acid (6% by weight). After mixing vigorously using a vortex mixer, the sample was centrifuged for 3 min at 3600 *g*, and

filtered through a Millipore filter. A l-ml aliquot of this solution was added to 1 ml of tri-N-octylamine—freon 113(1,1,2-trichlorotrifluoroethane) (0.5 *M*). After mixing 3—4 min and centrifuging for 3 min at 3600 *g*, the top layer was withdrawn and stored at −4°.

To determine the efficiency of recovery, the extraction procedure was carried out on 5 samples of serum to which known quantities of Ado were added. Recovery of Ado averaged 97%.

RESULTS

Separation of adenosine

Fig. 1A shows the resolution of Ado from 6 other nucleosides (Guo, Ino, Xao, Cyd, Urd, dThyd). These 6 nucleosides eluted within 5 min while the Ado peak is completely resolved. In the chromatogram of Fig. 1B, Ado and the other 6 nucleosides were co-injected with 5 nanomoles each of the bases of these 7 nucleosides. In Fig. 1C, 5 nanomoles of each of the mono-, di- and triphosphate nucleotides of these 7 nucleosides were co-injected with both the nucleosides and bases. These nucleic acid components, which may be found in blood extracts, did not interfere with the Ado peak.

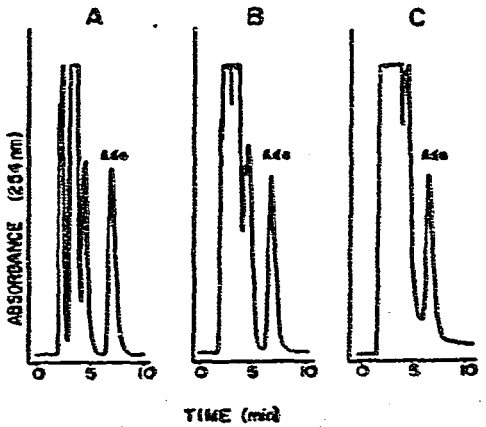

Fig. 1. (A) Separation of 5 nanomoles each of the 7 nucleosides cytidine, uridine, thymidine, guanosine, inosine, xanthosine and adenosine (Ado). (B). Co-injection of 7 nucleosides with 5 nanomoles each of the bases of these 7 nucleosides. (C) Separation of Ado from 5 nanomoles each of the nucleosides, the bases and the mono-, di- and tri-phosphate nucleotides of the 7 nucleosides. Column packing, μBondapak C_{18}; temperature, ambient; detector sensitivity, 0.02 a.u.f.s.; integrator setting 32. Eluent, anhyd. methanol—0.007 *F* KH_2PO_4 (pH 5.8) (10:90). Flow-rate, 2,0 ml/min.

Sensitivity and reproducibility

The lower limit of detection for Ado in a given chromatographic system will depend upon the detector sensitivity, noise levels and column efficiency. In the systems used for this study, 50 picomoles of Ado was readily detected (Fig. 2A). The peak shape remained constant up to 100 nanomoles (Fig. 2B). Excellent reproducibility of retention times and area was obtained. The relative

standard deviation of the retention time for 5 consecutive injections of standard solutions of Ado averaged 0.13%, while the peak area precision averaged 0.46%.

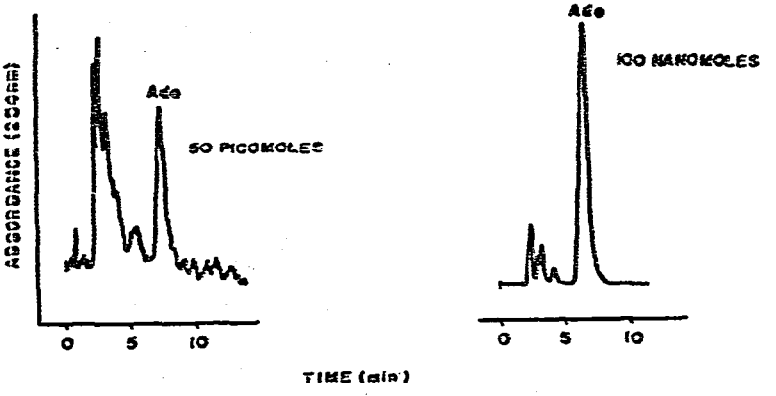

Fig. 2. (A) 5.0×10^{-11} moles (50 picomoles) of (Ado). Integrator setting, 1. (B) 1.0×10^{-9} moles (100 nanomoles) of Ado. Integrator setting, 512; further conditions as in Fig. 1.

Applications

Working in conjunction with Dr. Hilaire Meuwissen of Albany Medical College of Union University, we studied blood samples from a patient suffering from an adenosine deaminase deficiency with severe immunological defects along with normal control subjects. No free Ado was detected in normal human whole blood, erythrocytes or serum. However, in 3 serum samples taken at different time periods from the patient with the enzyme deficiency, 45 and 55 picomoles of Ado were readily detected. Two of these serum samples (Nos. 7 and 8) are shown in Fig. 3. Sample No. 10 of Fig. 3 shows a comparable serum sample of a control subject in which no Ado is found.

For positive identification of the peak with the retention time of Ado in the biological samples two techniques were used: standard addition and the enzymatic peak-shift. For the standard addition method, 50 picomoles of Ado were co-injected with the cell sample (Fig. 4B). In the enzymatic peak-shift technique, adenosine deaminase, which catalyzes the conversion of Ado to Ino, was used (Fig. 4C). With both standard solutions and cell extracts, the Ado was quantitatively converted to Ino. In addition to identifying the Ado, this technique showed that the Ado eluted free of any other UV-absorbing compound in serum; thus accurate quantitation of the Ado could be achieved with no errors caused by co-eluting compounds.

DISCUSSION

This analysis of adenosine using reversed-phase HPLC offers advantages over methods previously used. Rapid analyses can be achieved at ambient temperatures with sensitivity, selectivity and efficiency. The results are quantitative and other UV-absorbing constituents in cellular extracts do not interfere with

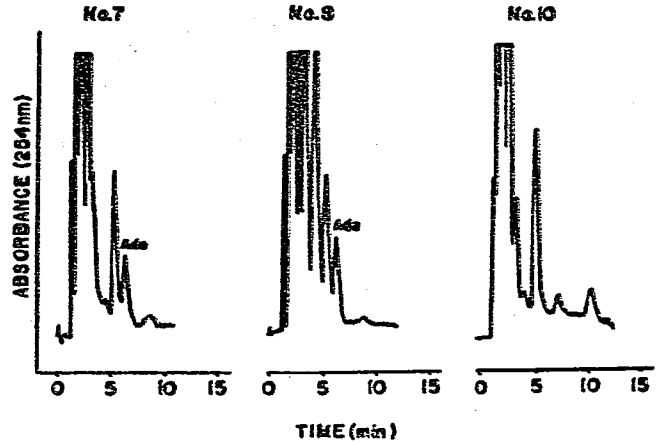

Fig. 3. Samples Nos. 7 and 8, 50 µl of serum extract from two patients suffering from adenosine deaminase deficiency. 45 and 55 picomoles are contained under the adenosine peak of sample numbers 7 and 8 respectively. Sample No. 10 shows the injection of 50 µl of serum extract from a control patient. Integrator setting, 2; further chromatographic conditions as in Fig. 1.

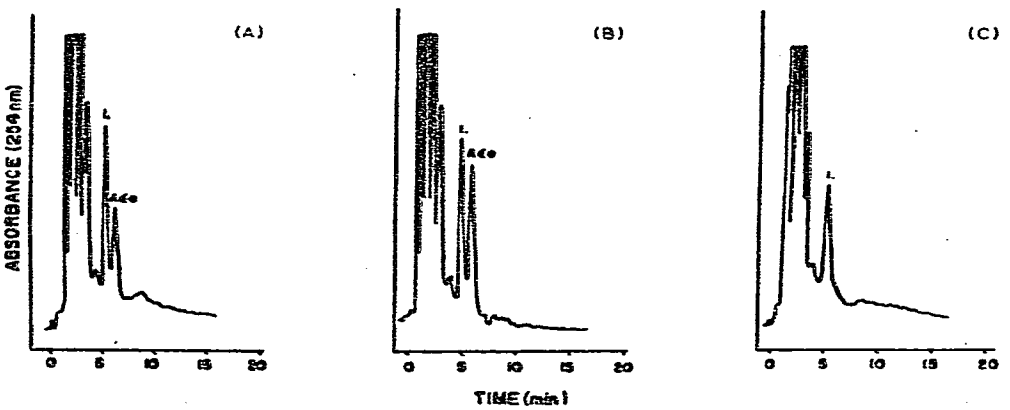

Fig. 4. Identification of Ado.(A) 50 µl of serum extract from an adenosine deaminase patient. (B) Co-injection of 50 picomoles of Ado with the serum extract. (C) Original serum extract after reaction with the enzyme, adenosine deaminase. Conditions as in Fig. 3; Peak 1, not identified.

the adenosine analysis. Minimal sample preparation is required prior to chromatography. Since the isocratic elution mode was used, no re-equilibration time was required between analyses. Thus the overall analysis time was shorter than the time necessary for an analysis in which gradient elution is used. In additon, with isocratic elution, it is easier to automate the procedure and therefore this method has potential for use in the clinical laboratory with a large number of samples. Furthermore, because the eluent is composed of a very weak salt solution and methanol, the solvent can readily be removed after fractions are

collected. Using the enzymic peak-shift together with retention times and the standard addition method, the adenosine peak can be positively identified. The enzyme used in this peak-shift, adenosine deaminase, is commercially available and is inexpensive. We found that the reversed-phase chemically bonded microparticle columns were very stable and the chromatograms highly reproducible over a period of time. Columns have been used continuously in our laboratory for 6 months with no loss in efficiency. However, if the retention time or the resolution of adenosine is found to shift with time, it is possible that organics from the eluent or the samples are being partitioned into the stationary phase. In such cases, the column can be cleaned with methanol or some moderately polar solvent. It should be noted that retention characteristics may vary slightly from batch to batch or from different manufacturers. Thus the conditions for this analysis may be used as a guide-line, but conditions should be optimized for each column to obtain the best analysis.

Although the results presented here were achieved using a Waters μBondapak C_{18} column, similar results were obtained in our laboratory using Whatman Partisil 10-ODS and DuPont Zorbax™ ODS columns on a DuPont Model 830 liquid chromatograph.

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REFERENCES

1 C.G. Horvath, B.A. Preis and S.R. Lipsky, Anal. Chem., 39 (1967) 1422.
2 P.R. Brown, J. Chromatogr., 52 (1970) 257.
3 P.R. Brown, High Pressure Liquid Chromatography, Biochemical and Biomedical Applications, Academic Press, New York, 1973.
4 P.R. Brown, R.E. Parks, Jr. and J. Herod, Clin. Chem., 19 (1973) 919.
5 E.M. Scholar, P.R. Brown, R.E. Parks, Jr. and P. Calabresi, Blood, 41 (1973) 927.
6 P.R. Brown and R.E. Parks, Jr., Anal. Chem., 44 (1973) 1072.
7 R.E. Parks, Jr. and P.R. Brown, Biochemistry, 12 (1973) 3294.
8 C.D. Scott, Clin. Chem., 14 (1968) 521.
9 C.D. Scott, J. Attril and N.G. Anderson, Proc. Soc. Exp. Biol. Med., 125 (1967) 181.
10 C.A. Burtis and K.S. Warren, Clin. Chem., 14 (1968) 290.
11 C.A. Burtis, J. Chromatogr., 52 (1970) 97.
12 J.E. Mrochek, D.D. Chilcote and S.R. Dinsmore, Liquid Chromatographic Analysis of Urinary Nucleosides in Normal and Malignant States, American Chemical Society Meeting, August, 1973.
13 C.W. Gehrke, R.W. Zumwalt, K.C. Kuo and T.P. Walker, Symposium on Chromatographic Analysis of Biologically Important Compounds, American Chemical Society Meeting, August, 1975.
14 R.M. Berne and R. Rubio, Circ. Res., Suppl. III, 34 & 35 (1974) 109.

15 R. Rubio and R.M. Berne, Circ. Res., 25 (1969) 407.
16 K. Ishii and H. Green, J. Cell Sci., 13 (1973) 1.
17 H. Green, in H.J. Meuwissen et al. (editors), Combined Immunodeficiency Disease and Adenosine Deaminase Deficiency, Academic Press, New York, 1975.
18 C. Bishop and D.M. Surgenor, The Red Blood Cell, Academic Press, New York, 1964, pp. 173—175.
19 C.G. Horvath and S.R. Lipsky, Anal. Chem., 41 (1969) 1227.
20 P.R. Brown, S. Bobick and F.L. Hanley, J. Chromatogr., 99 (1974) 587.
21 N.G. Anderson, Anal. Biochem., 4 (1962) 269.
22 N.G. Anderson, J.G. Green, M.L. Barber and L.C. Ladd, Anal. Biochem., 6 (1963) 153.
23 E.M. Scholar, P.R. Brown and R.E. Parks, Jr., Cancer Res., 32 (1972) 259.
24 P.R. Brown, R.P. Agarwal, J. Geli and R.E. Parks, Jr., Compr. Biochem. Physiol., 43 (1972) 891.
25 M. Uziel, C.K. Koh and W.E. Cohn, Anal. Biochem., 25 (1968) 77.
26 R.P. Singhali and W.E. Cohn, Anal. Biochem., 45 (1972) 585.
27 W.E. Cohn, Science, 109 (1949) 377.
28 P. Reichard and B. Estbarn, Acta Chem. Scand., 4 (1950) 1047.
29 C.F. Crampton, F.R. Fraenkel, A.M. Benson and A. Wade, Anal. Biochem., 1 (1960) 249.
30 M. Uziel and C. Koh, J. Chromatogr., 59 (1971) 188.
31 F. Murakami, S. Rokushika and H. Hatano, J. Chromatogr., 53 (1970) 584.
32 C.D. Scott, R.L. Jolley and W.F. Johnson, Amer. J. Clin. Pathol., 53 (1970) 701.
33 J.E. Mrochek, W.C. Butts, W.C. Rainey and C.A. Burtis, Clin. Chem., 17 (1971) 72.
34 C.D. Scott, D.D. Chilcote and N.E. Lee, Anal. Chem., 44 (1972) 85.
35 F.R. Blattner and H.P. Erickson, Anal. Biochem., 18 (1967) 220.
36 H.J. Breter and R.K. Zahn, Anal. Biochem., 54 (1973) 346.
37 M. Carrara and G. Bernerdi, Biochim. Biophys. Acta, 155 (1968) 1.
38 J.X. Khym, Clin. Chem., 21 (1975) 1245.
39 D.C. Tormey, T.P. Waalkes, D. Ahmann, C.W. Gehrke, R.W. Zumwalt, J. Snyder and H. Hansen, Cancer, 35 (1975) 1095.
40 T.P. Waalkes, C.W. Gehrke, R.W. Zumwalt, S.Y. Chang, D.B. Lakings, D.C. Tormey, D.L. Ahmann and C.G. Moertel, Cancer, 36 (1975).