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Note

Simple and rapid high-performance liquid chromatographic method for analysis of nucleosides in biological fluids

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Patients with severe combined immunodeficiency (adenosine deaminase deficiency variant) lack both T- and B-cell function and have been reported to have elevated plasma dAdo* levels [1—4]. The adequacy of enzyme replace ment therapy appears to correlate with decreases in plasma dAdo concentrations [4, 5]. Recent in vitro studies have demonstrated a marked enhancement of dAdo toxicity to lymphoid cells, when ADA activity is inhibited by specific ADA inhibitors [6]. The neurotoxic complications following accumulation of Ado and dAdo in the plasma and cerebrospinal fluid of patients treated with a potent ADA inhibitor, deoxycoformycin [7, 8], and the neurotoxicity caused by dAdo and Ado in experimental animals [9—11] suggest that these nucleosides might play a role as neurotransmitters. Another adenosine analogue, Ara-A, a useful antiviral agent [12, 13], is now being evaluated in patients for its antileukemic properties [14—16]. These observations demonstrate the need for rapid, reproducible and sensitive methods for separation and quantification of Ado, dAdo, Ara-A and their metabolites in body fluids.

*Abbreviations used: ADA = adenosine deaminase (adenosine aminohydrolase, E.C. 3.5.4.4); Ado = adenosine; dAdo = 2'-deoxyadenosine; Ara-A = 9-β-D-arabinofuranosyladenine (arabinosyladenine); Ara-Hyp = 9-β-D-arabinofuranosylhypoxanthine (arabinosylhypoxanthine); DCF = deoxycoformycin; HPLC = high-performance liquid chromatography.

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Recently, several methods have been reported for the separation of nucleosides [17—31]; except for two [30, 31], most of these methods require the use of either large volumes of body fluids, tedious protein precipitation procedures and/or radioactive chemicals. Furthermore, the separation of ribosyl-, deoxyribosyl- and arabinosylpurines present in a mixture has not met with success. In order to separate them one has to employ special techniques, e.g., periodation or use of borate. The method we report here is simple, rapid and reproducible, and has been applied successfully to quantify Ado, dAdo, Ara-A and their degradation products in clinical samples of body fluids.

EXPERIMENTAL

Chemicals

Purine bases and nucleosides were purchased from P.L. Biochemicals (Milwaukee, WI, U.S.A.). Ara-A was a gift from Parke Davis (Ann Arbor, MI, U.S.A.). Adenosine deaminase (specific activity 195 units/mg protein) was obtained from Sigma (St. Louis, MO, U.S.A.). Potassium dihydrogen phosphate (analytical-reagent grade) was obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.) and HPLC-grade methanol from Fisher Scientific (Fairlawn, NJ, U.S.A.). All other reagents were of the highest grade available through commercial suppliers.

Equipment

Throughout the study we used the following equipment: Altex Model 312A liquid chromatograph (Altex Scientific, Berkeley, CA, U.S.A.), equipped with two pumps (Model 110), a microprocessor solvent programmer (Model 410), a UV—visible variable-wavelength detector (Model 155-100), a syringe injector valve with a 100-μl sample loop, a reporting integrator (Model 3380A; Packard, Avondale, PA, U.S.A.) and a reversed-phase Ultrasphere-ODS 5-μm column (150 × 4.6 mm; Altex).

Methods

The eluents for HPLC were 10 m*M* KH_2PO_4 (solution A) and 30% methanol in 10 m*M* KH_2PO_4 (solution B), pH 4.9. Each solution was prepared fresh daily by diluting from a 500 m*M* KH_2PO_4 stock solution (which was stored at 4°C) and filtering through an HA 0.45-μm Millipore filter (Millipore, Bedford, MA, U.S.A.).

Plasma, cerebrospinal fluid and urine samples were prepared by filtering under centrifugal force through Amicon Centriflo CF25 ultrafiltration membrane cones (Amicon, Lexington, MA, U.S.A.) at 1100 *g* for 20 min. This method is rapid for deproteinization and gives excellent recoveries of nucleosides. Excellent recoveries of inosine and xanthosine from human serum by use of ultrafiltration have also been reported by Hartwick et al. [30]. The filtered samples were either analyzed immediately or stored at —20°C.

Aliquots of 10—20 μl were injected directly for HPLC analysis. The elutions were performed by using a linear gradient of 10—30% of solution B in solution A, achieved in 15 min at a flow-rate of 1 ml/min. Absorbance was monitored at 254 nm.

Evaluation of recovery of Ara-A and Ara-Hyp from human plasma. Plasma samples, with or without 2 μM deoxycoformycin, an inhibitor of ADA, were incubated for 15 min prior to the addition of known amounts of Ara-Hyp and Ara-A. Following a 20-min incubation at room temperature the samples were filtered through Amicon Centriflo ultrafiltration membranes as described above. The ultrafiltrates were then analyzed by HPLC and the amounts of Ara-A and Ara-Hyp quantified.

Confirmation of nucleoside identity of peak-shift method. The peak-shift method was employed for additional and definitive identification of the peaks having the retention times of adenosine, deoxyadenosine and Ara-A. The fractions corresponding to these peaks on HPLC were collected separately and each was treated with one unit of ADA. After incubation for 15—20 min at room temperature the proteins were removed either by filtration through Amicon Centriflo ultrafiltration membranes or by heating the mixture for 1 min in a boiling water-bath, cooling on ice and centrifugation. The samples were then re-analyzed by HPLC. Disappearance of the original peaks and appearance of peaks corresponding to their deaminated products established the identity of these compounds.

RESULTS AND DISCUSSION

Chromatographic separation

Fig. 1 presents an HPLC profile of a number of nucleosides and purine bases. The separation of Ara-A and its metabolite, Ara-Hyp, is shown in Fig. 2. Ado, dAdo and Ara-A are clearly separated. Furthermore, the metabolic

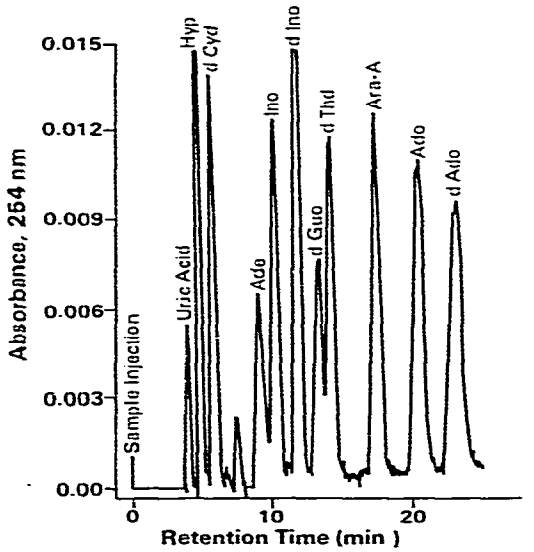

Fig. 1. Separation of a standard mixture of nucleosides and bases by HPLC. Injection volume, 10 μl; column, Ultrasphere ODS; flow-rate, 1 ml/min. Peaks: uric acid, hypoxanthine (Hyp), deoxycytidine (dCyd), adenine (Ade), inosine (Ino), deoxyinosine (dIno), deoxyguanosine (dGuo), thymidine (dThd), arabinosyl adenine (Ara-A), adenosine (Ado) and deoxyadenosine (dAdo).

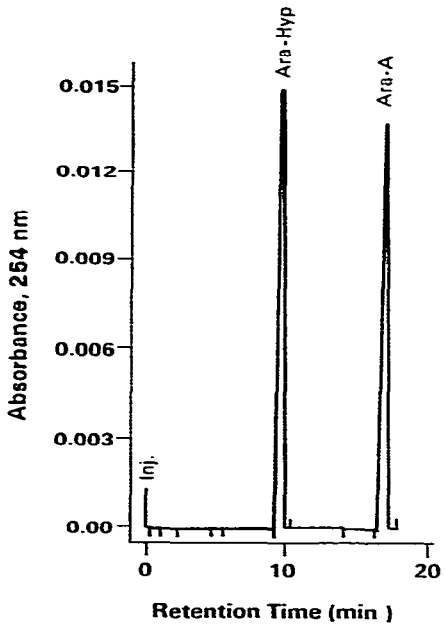

Fig. 2. HPLC separation of arabinosylhypoxanthine (Ara-Hyp) and arabinosyladenine (Ara-A). A 10-μl aliquot of a mixture containing 13.0 μg/ml each of Ara-A and Ara-Hyp was injected.

products of these nucleosides are also resolved in a single run. For example, Ado, Ino, dIno, Ara-Hyp, adenine, hypoxanthine and uric acid are all resolved. Although not illustrated, this procedure can also be used to resolve guanosine from deoxyguanosine and other nucleosides such as deoxycoformycin (Table I).

The retention times show very little day-to-day variation. The small differences did not cause either overlapping or alterations in the pattern illustrated

TABLE I

RETENTION TIMES OF SOME BASES, RIBONUCLEOSIDES, DEOXYRIBONUCLEOSIDES AND ARABINOSIDES

Compound	Retention time (min)*	Compound	Retention time (min)*
Uric acid	3.88 ± 0.61 (85)	Deoxyinosine	11.75 ± 0.76 (85)
Hypoxanthine	4.64 ± 0.59 (85)	Guanosine	11.90 ± 0.45 (5)
Deoxycytidine	5.64 ± 0.64 (85)	Deoxyguanosine	13.13 ± 0.76 (85)
N^1-Methyladenosine	6.67 (2)	Thymidine	14.10 ± 0.83 (85)
Deoxycoformycin	7.91 ± 0.62 (3)	5-Bromodeoxyuridine	17.01 (2)
Adenine	8.40 ± 1.09 (79)	Arabinosyladenine	17.24 ± 0.64 (45)
Arabinosylhypoxanthine	9.61 ± 0.31 (26)	Adenosine	20.00 ± 1.32 (85)
Inosine	10.18 ± 0.64 (85)	Deoxyadenosine	22.38 ± 1.66 (85)

*Mean ± S.D. (n).

in Fig. 1. These small differences were due to the mechanical adjustment rather than to changes in resolution, and therefore remained constant and were reflected in all profiles on that particular day. Therefore, a common practice has been to run a mixture of standards once in the morning and at the end of the day. Table I summarizes the retention times of all standard compounds examined in runs performed over a 2-year period.

Maintenance of the column

After prolonged use, aberrant separation profiles were sometimes obtained. This problem was usually corrected by washing the column for 15—30 min in the following order: first distilled water, then 1 m*M* phosphoric acid, followed by distilled water, 70% (v/v) methanol and distilled water. All solutions were filtered through Millipore filters (HA, 0.45 μm). At the end of the day the column was flushed with 20—30 ml of 30% methanol in 10 m*M* KH_2PO_4. Storage of the column, when it is not in use for more than 3 days, in 70% methanol prolongs its useful life. In our laboratory, the same column has been in use for more than 2 years for analyzing over 2000 samples. The quality and retention times of the peaks have been excellent.

Calibration graphs and accuracy

The relationship between the concentration and the peak area of nucleosides was linear over a wide range of concentrations. Using 100-μl samples, we could detect as low as 10 pmole of Ara-A (or Ado or dAdo). This sensitivity is similar to that recently reported with another HPLC method, which used radioactive nucleosides and protein precipitation [26].

The recoveries of Ara-A and Ara-Hyp added to plasma were 95 ± 4% and 101 ± 2.5%, respectively. However, in the absence of DCF the recovery of exogeneously added Ara-A was slightly lower (83%) and there was correspondingly a slightly larger amount of Ara-Hyp (110%) resulting from deamination of Ara-A. This suggests that blood samples for the determination of Ara-A or other adenosine analogues must be collected in tubes containing DCF. The DCF will inhibit red blood cell ADA, which would also degrade Ara-A following sample collection. In addition, keeping the samples cold and removing the red blood cells from plasma as soon as possible could further minimize degradation of adenosine analogues.

Application

The application of this method to human samples of blood, urine and cerebrospinal fluid from a patient treated with deoxycoformycin and Ara-A is shown in Fig. 3. For example, the concentrations of dAdo (a natural nucleoside) are so low that the peaks of this nucleoside are undetectable in either plasma or urine of normal or leukemic subjects (not shown here). However, this nucleoside accumulates following treatment of patients with an ADA inhibitor, DCF (see left and right panels of Fig. 3 and refs. 7 and 8). An accumulation of dAdo has also been reported in the plasma and urine of patients with genetic deficiency of ADA [5]. Furthermore, treatment of leukemic patients with DCF and Ara-A caused accumulation of Ara-A in the plasma, urine and cerebrospinal fluid of the patients (Fig. 3). On treatment with

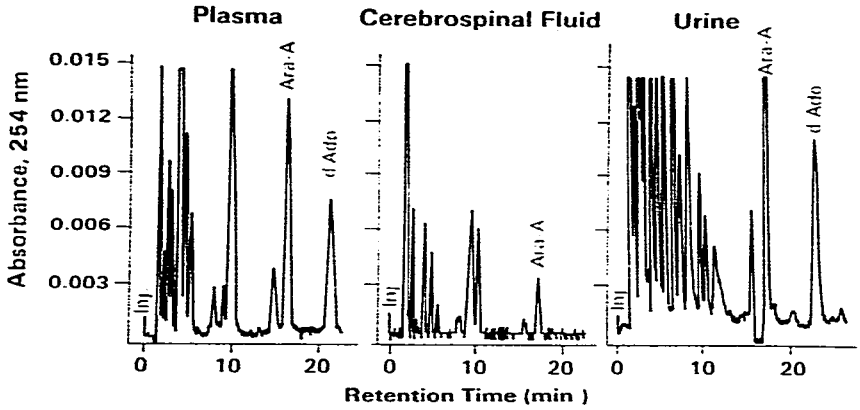

Fig. 3. HPLC profiles of plasma, cerebrospinal fluid and urine from a leukemic patient. The patient was treated with arabinosyladenine i.v. infusion (300 mg/m^2, 180 min) following a single i.v. dose of deoxycoformycin (15 mg/m^2). The samples were prepared as described under *Methods*. A 20-μl volume of the plasma and cerebrospinal fluid and 5 μl of urine were used for analysis.

Ara-A alone the drug was rapidly deaminated to Ara-Hyp and the peaks of Ara-A were undetectable (not shown here). The identification of dAdo and Ara-A was further confirmed by the peak-shift method. Details of pharmacokinetic studies of DCF and Ara-A combination will be published elsewhere.

The method presented here offers the advantage of rapid and simple preparation of samples of either plasma, urine or cerebrospinal fluid and uses volumes as small as 0.5 ml. There is no need to extract the nucleosides or to remove or modify sugar moieties to obtain adequate separation. In pharmacokinetic studies, when amounts of clinical material are small and large numbers of assays are required, the present method is very useful. We are presently using this method for measuring Ara-A, Ara-Hyp, Ado and dAdo in the body fluids of patients receiving treatment with Ara-A/deoxycoformycin. However, the method should be useful to other investigators performing similar studies. Further, with slight modification, this method has been adapted to resolve arabinosyl cytosine, arabinosyl uracil and fluorouracil and is being used in pharmacokinetic studies of these drugs.

This method has been used successfully to assay Ado and dAdo in the body fluids of patients treated with DCF [7, 8]. The method should also find use in monitoring the levels of purines and nucleosides in patients with defects of purine metabolism, e.g., hypoxanthine—guanine phosphoribosyl transferase (E.C. 2.4.2.8) deficiency (Lesch—Nyhan syndrome) [32], purine nucleoside phosphorylase (E.C. 2.4.2.1) deficiency [33] and severe combined immunodeficiency—ADA deficiency, etc.

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REFERENCES

1 E.R. Giblett, G. Anderson and F. Cohen, Lancet, ii (1972) 1067.
2 G.C. Mills, F.C. Schmalstieg, K.B. Trimmer, A.S. Goldman and R.M. Goldblum, Proc. Nat. Acad. Sci. U.S., 73 (1976) 2867.
3 H.A. Simmonds, A. Sahota, C.F. Potter and J.S. Cameron, Clin. Sci. Mol. Med., 54 (1978) 579.
4 R. Hirschhorn, V. Roegner, A. Rubinstein and P.S. Papageorgious, J. Clin. Invest., 65 (1980) 768.
5 R. Hirschhorn, P.S. Papageorgiou, H.H. Keserwala and L.T. Taft, N. Engl. J. Med., 303 (1980) 377.
6 D.A. Carson, J. Kay and J.E. Seegmiller, Proc. Nat. Acad. Sci. U.S., 74 (1977) 5677.
7 P.P. Major, R.P. Agarwal and D.W. Kufe, Cancer Chemother. Pharmacol., 5 (1981) 193.
8 P.P. Major, R.P. Agarwal and D.W. Kufe, Blood, 58 (1981) 91.
9 I. Haulica, L. Adabei, D. Brainisteanu and F. Topoliceanu, J. Neurochem., 21 (1973) 1019.
10 J.W. Phillis and J.P. Edstrom, Life Sci., 19 (1976) 1041.
11 J.W. Phillis, J.K. Kostopoulos, J.P. Edstrom and S.W. Ellis, in H.P. Baer and G.I. Drummond (Editors), Physiological and Regulatory Functions of Adenosine and Adenine Nucleotides, Raven Press, New York, 1979, p. 373.
12 F.M. Schabel, Jr., Chemotherapy (Basel), 13 (1968) 321.
13 C.A. Alford and R.J. Whitley, J. Infect. Dis., 133, Suppl. (1976) A101.
14 G.P. Bodey, J. Gottlieb, K.B. McCredie and E.J. Freireich, in D. Pavan-Langston, R.A. Buchanan and C.A. Alford (Editors), Adenine Arabinoside: An Antiviral Agent, Raven Press, New York, 1975. p. 281.
15 D.P. Gray, M.R. Grever, M.F.E. Siaw, M.S. Coleman and S.P. Balcerzak, Proc. Amer. Ass. Cancer Res., 22 (1981) 488.
16 W. Plunkett, R. Benjamin, L. Feun, M. Keating and E.J. Freireich, Proc. Amer. Ass. Cancer Res., 22 (1981) 177.
17 P.R. Brown, S. Bobick and F.L. Heinley, J. Chromatogr., 99 (1974) 587.
18 M. Uziel, L.H. Smith and S.A. Taylor, Clin. Chem., 22 (1976) 1451.
19 R.A. Hartwick and P.R. Brown, J. Chromatogr., 143 (1977) 383.
20 J.F. Kuttesch, F.C. Schmalstieg and J.A. Nelson, J. Liquid Chromatogr., 1 (1978) 97.
21 U.E. Honegger, S.S. Bogdanov and P.R. Bally, Anal. Biochem., 82 (1977) 262.
22 A.M. Krstulovic, P.R. Brown and D.M. Roise, Anal. Chem., 49 (1977) 2237.
23 H.G. Schneider and A.J. Glazko, J. Chromatogr., 139 (1977) 370.
24 B. Bakay, E. Nissinen and L. Sweetman, Anal. Biochem., 86 (1978) 65.
25 A. McBurney and T. Gibson, Clin. Chim. Acta, 102 (1980) 19.
26 C.A. Koller, P.L. Stetson, L.D. Nichamin and B.S. Mitchell, Biochem. Med., 24 (1980) 179.
27 E.C. Reynolds and L.R. Finch, Anal. Biochem., 82 (1977) 591.
28 A.M. Krstulovic, R.A. Hartwick and P.R. Brown, Clin. Chim. Acta, 97 (1979) 159.
29 R. Hartwick, A. Jeffries, A. Krstulovic and P.R. Brown, J. Chromatogr. Sci., 16 (1978) 427.
30 R.A. Hartwick, D. VanHaverbeke, M. McKeag and P.R. Brown, J. Liquid Chromatogr., 2 (1979) 725.
31 S.P. Assenza and P.R. Brown, J. Chromatogr., 181 (1980) 169.
32 M. Lesch and W.L. Nyhan, Amer. J. Med., 36 (1964) 561.
33 E.R. Giblett, A.J. Ammann, D.W. Wara, R. Sandman and L.K. Diamond, Lancet, i (1975) 1010.