Journal of Chromatography, 183 (1980) 499—504
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 624

Note

Identification of adenosine and eight modified adenine nucleosides using reversed-phase high-performance liquid chromatography and enzymatic peak shift with adenosine deaminase

HOWARD RATECH and G. JEANETTE THORBECKE

Department of Pathology, New York University School of Medicine, New York, NY 10016 (U.S.A.)

and

ROCHELLE HIRSCHHORN*

Department of Medicine, New York University Medical Center, 550 First Avenue, New York, NY 10016 (U.S.A.)

(First received February 5th, 1980; revised manuscript received April 22nd, 1980)

The modified nucleosides have been increasingly studied by high-performance liquid chromatography (HPLC) [1—17]. They are of interest as indicators of RNA metabolism [1—3, 17] and because some are elevated in the urines of patients with cancer [4, 5, 18]. It is the purpose of this report to show that the naturally occurring modified adenine nucleosides (and 3′-O-methyladenosine) can be identified by high-performance reversed-phase chromatography which has been optimized for the separation of most nucleosides [9, 13]. The basis for this identification is the enzyme peak shift in which the retention times are determined, both of the sample and of the N^6-deaminated product, after incubation with adenosine deaminase (ADA) [7, 8].

MATERIALS

Adenosine deaminase was prepared from chicken liver by the method of Ma and Fisher [19] with the following modifications: A Sephacryl S-200 column was used for the initial chromatography instead of a Sephadex G-150 column. Additionally, the 35,000 molecular weight ADA fractions obtained were concentrated by ammonium sulphate precipitation, passed through a Sephadex G-75 column and fractions containing 35,000 molecular weight ADA reconcentrated. This 35,000 molecular weight ADA, used for the HPLC enzymatic

0378-4347/80/0000-0000/$02.25

analysis, had no detectable contaminating purine nucleoside phosphorylase activity as determined spectrophotometrically.

1-Methyladenosine, 3'-O-methyladenosine, N^6,N^6-dimethyladenosine, N^6-isopentenyladenosine, N^6-2'-O-dimethyladenosine, and 1-methylinosine were purchased from P & L Biochemicals (Milwaukee, WI, U.S.A.). Adenosine, 2'-deoxyadenosine, inosine, and 2'-deoxyinosine were purchased from Sigma (St. Louis, MO, U.S.A.). Other reagents used were the highest grade obtainable: monobasic ammonium dihydrogen phosphate, Fisher Scientific (Fairlawn, NJ, U.S.A.), methyl alcohol, Burdick and Jackson Labs. (Muskegon, MI, U.S.A.) and Tris (Base) Ultra Pure, Schwarz/Mann (New York, NY, U.S.A.). Water used was passed through an Ultra Pure waters system, Hydro Service and Supplies (Durham, NC, U.S.A.). Type 1 calf intestinal ADA was obtained from Sigma.

METHODS

Standard compounds were dissolved in 0.01 *M* Tris—HCl buffer, pH 7.0, at a concentration of 10^{-4} *M*. Incubation of nucleosides with chicken liver ADA varied from 15 min to 24 h at 37°C. Aliquots were removed at different time intervals until there was 95—100% conversion of the substrate (except for N^6, N^6-dimethyladenosine, which was a very poor substrate). In order to analyze a complex mixture, nucleosides can be incubated with 50 times the amount of enzyme necessary to totally convert adenosine in one hour. Similar results can be obtained with commercially available calf intestinal ADA (Sigma) using 17.4 U/1.0 ml for 1 h at 37°C.

Analyses were performed on a reversed-phase column [μBondapak C_{18}, Waters Assoc. (Milford, MA, U.S.A.)] at room temperature using a modification of published methods [9, 15]. Two single-piston high-pressure pumps (Constametric I and II, Laboratory Data Control, Riviera Beach, FL, U.S.A.) delivered solvents in a linear gradient from 0—40% methanol in 0.01 *F* $NH_4H_2PO_4$, pH 5.5, during 60 min at a final constant flow-rate of 1.5 ml/min. Samples (25—100 μl) were injected (Rheodyne 7120 syringe loading sample injection valve, Laboratory Data Control) and the effluent monitored with a variable-wavelength ultraviolet detector at 260 nm with a 10-μl flow cell (modified Spectromonitor 1, Laboratory Data Control).

RESULTS AND DISCUSSION

In preliminary experiments, when the various adenine nucleosides were incubated with a relatively crude preparation of ADA in the presence of phosphate buffer, two different products were often generated. These were thought to result from the action of contaminating nucleoside phosphorylase, which can further metabolize the deaminated nucleosides to the respective base and ribose-1-phosphate or to a kinase yielding a phosphorylated derivative. Both of these reactions require phosphate [20]. Therefore the ADA was further purified and incubations with ADA were carried out in Tris buffer. Under these conditions a single product was formed for each compound investigated.

Chicken ADA (molecular weight 35,000) has a broad spectrum of activity in its ability to N^6-deaminate adenine nucleosides with modifications in the

base or sugar moieties [21—23]. All eight modified nucleosides served as substrates for chicken liver ADA as assayed by HPLC [23]. Table I lists the retention times of adenosine, seven naturally occurring adenine nucleosides (and 3'-O-methyladenosine) and their corresponding inosine products after treatment with ADA. All of the modified adenine nucleosides eluted separately except for 3'-O-methyladenosine and N^6-methyladenosine, which co-eluted. Incubation of either adenosine, N^6-methyladenosine, N^6,N^6-dimethyladenosine or N^6-isopentenyladenosine with ADA all resulted in the appearance of the same product, which co-eluted with authentic inosine. Incubation of 1-methyladenosine gave rise to 1-methylinosine and of 2'-deoxyadenosine to 2'-deoxyinosine, as determined by co-elution with authentic 1-methylinosine and 2'-deoxyinosine. The N^6-2'-O-dimethyladenosine and 2'-O-methyladenosine both gave rise to a product with the same retention time. It was presumed that 2'-O-methylinosine was their single corresponding N^6-deaminated product. Similarly, 3'-O-methyladenosine was presumed to yield 3'-O-methylinosine. In support of this conclusion, the area of the peaks of the products generated was approximately one half that of the substrate, similar to the absorption ratios of adenosine and inosine at 260 nm. Thus, under these conditions, all of the N^6-deaminated products (inosine, 2'-deoxyinosine, 1-methylinosine, 2'-O-methylinosine and 3'-O-methylinosine) had characteristic, non-overlapping retention times. (It should be noted that 3'-O-methyladenosine stored at −20°C for several months in water contained trace amounts of an unidentified contaminant that eluted at 30.0 min and was not sensitive to treatment by ADA.)

Six of the eight compounds tested each had unique retention times. While N^6-methyladenosine and 3'-O-methyladenosine had identical retention times, their respective deaminated reaction products, inosine and 3'-O-methylinosine were quite widely separated. However, in biological samples other compounds could have similar elution times. Therefore, application of the peak shift method [7, 9] using ADA allows for identification of an ADA-sensitive compound and of the deaminated product.

TABLE I

RETENTION TIMES OF ADENOSINE AND EIGHT MODIFIED ADENINE NUCLEOSIDES AND OF THEIR SINGLE PRODUCTS GENERATED AFTER INCUBATION WITH ADA

See Materials and Methods for preparation of chicken liver adenosine deaminase (molecular weight 35,000), for enzymatic assay conditions and for HPLC system used.

Substrate	Retention time (min)	Product	Retention time (min)
1-Methyladenosine	6.7	1-Methylinosine	12.0
Adenosine	16.1	Inosine	8.6
2'-Deoxyadenosine	17.7	2'-Deoxyinosine	10.4
2'-O-Methyladenosine	21.4	2'-O-Methylinosine	13.2
3'-O-Methyladenosine	24.2	3'-O-Methylinosine	14.8
N^6-Methyladenosine	24.2	Inosine	8.6
N^6-2'-O-Dimethyladenosine	28.8	2'-O-Methylinosine	13.2
N^6,N^6-Dimethyladenosine	33.9	Inosine	8.6
N^6-Isopentenyladenosine	55.5	Inosine	8.6

The applicability of the enzyme peak shift technique to detection of modified adenine nucleosides in biological samples is illustrated in Fig. 1. Peaks with retention times of 1-methyladenosine and 2'-deoxyadenosine are seen in this chromatogram of urine from a neonatal mouse which had been injected with deoxycoformycin. Following incubation of the urine sample with ADA (lower panel) the 1-methyladenosine and 2'-deoxyadenosine peaks are no longer visible but two new peaks have appeared with the retention times of 1-methylinosine and 2'-deoxyinosine.

Application of this method to identification of all of the compounds in biological samples may require preliminary separative procedures such as anion-exchange [24] or boronate columns [5]. Unambiguous determination may on occasion require collection of relevant substrate peak, concentration, treatment of this isolated peak with ADA, and rechromatographing to determine product

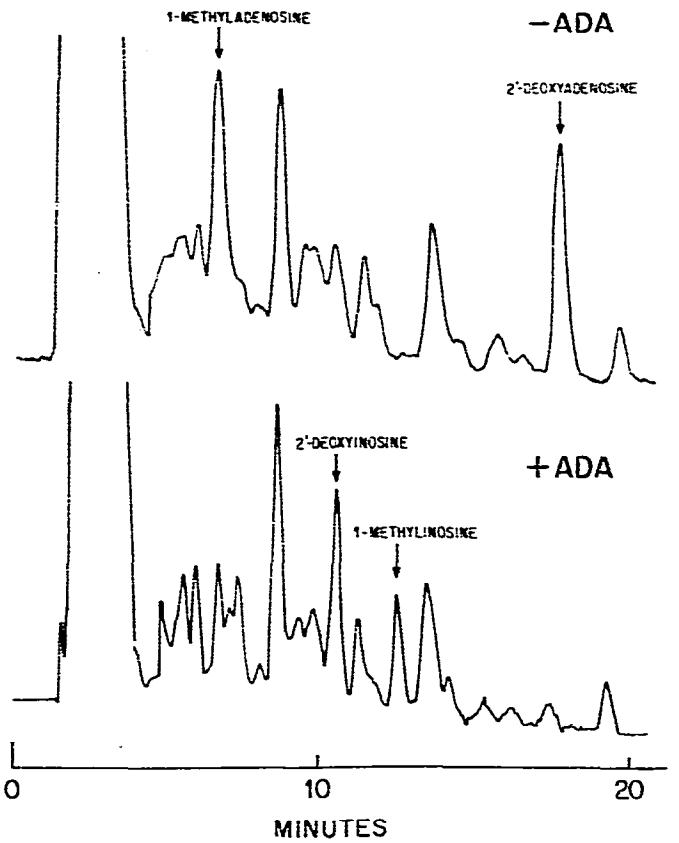

Fig. 1. Demonstration of enzymatic peak shift technique for identification of 1-methyladenosine and 2'-deoxyadenosine in whole neonatal mouse urine. The mouse had been treated with deoxycoformycin, an ADA inhibitor, 24 h prior to collection of the urine. Two peaks, eluting at 6.4 and 17.7 min, the retention times of 1-methyladenosine and 2'-deoxyadenosine respectively, are seen in the upper panel but not following treatment of the urine with ADA (lower panel). Two peaks eluting at 12.5 and 10.1 min, the retention times of 1-methylinosine and 2'-deoxyinosine respectively, appear in the lower chromatogram following treatment of the urine with ADA but are not seen in the untreated urine (upper panel).

retention time without the presence of interfering compounds that might have been present in the original complex sample mixture.

Several modified and unmodified adenine nucleosides and bases have previously been analysed using HPLC (Table II). We have identified all of these modified nucleosides, except for N^6-isopentenyl-2-methylthioadenosine in the

TABLE II

ADENINE NUCLEOSIDES ANALYZED BY HPLC

Compound	Column	Reference
1-Methyladenosine	Cation	6
	Reversed phase	5,*
Adenosine	Cation	6
	Reversed phase	5, 7—9, 11, 13, 14,*
2'-Deoxyadenosine	Cation	22
	Reversed phase	14,*
N^6-Methyladenosine	Cation	6
	Reversed phase	13,*
N^6-2'-O-Dimethyladenosine	Reversed phase	*
N^6,N^6-Dimethyladenosine	Cation	6
	Reversed phase	*
N^6-Isopentenyladenosine	Cation	15
	Reversed phase	10,*
N^6-Isopentenyl-2-methylthioadenosine	Cation	15
	Reversed phase	10,*
2'-O-Methyladenosine	Reversed phase	*
3'-O-Methyladenosine	Reversed phase	*

*This paper.

system used here, and additionally have identified 2'-O-methyladenosine, 3'-O-methyladenosine and N^6-2-O-dimethyladenosine and their respective deaminated products. Analysis of modified adenine nucleosides using reversed-phase columns would appear to be most advantageous for application to biological materials, since these compounds (except for 1-methyladenosine) are relatively retarded and therefore elute after most of the other UV absorbing materials. As more attention is directed to analyzing tRNA metabolism and the modified nucleosides found in the urine of patients with neoplastic and metabolic diseases the importance of identifying members of this class of compounds will increase.

In conclusion, identification of the modified adenine nucleosides has been demonstrated on reversed-phase chromatography on the basis of the differential retention times of the nucleosides and/or the reaction products generated after treating the nucleoside sample mixture with ADA. This extends the usefulness of the previously described enzymatic peak shift technique for identifying adenosine [7, 9]. It should prove of value in further identifying the modified adenine nucleosides in polynucleotide metabolism and in complex biological tissue and fluid samples. Preliminary investigations suggest that this method can indeed be applied to body fluids.

ACKNOWLEDGEMENTS

This investigation was supported by NIH A1 10343, CA 16247 and National Foundation No. 6—4. H. Ratech was supported by a Clinical Fellowship of the American Cancer Society, a Leopold Schepp Foundation Scholarship, and NC 1 CA 09161.

REFERENCES

1 M. Kratovich and B.A. Roe, J. Chromatogr., 155 (1978) 407.
2 B.A. Roe, K. Marcu and B. Dudock, Biochim. Biophys. Acta, 319 (1973) 25.
3 R.P. Singhal, Biochim. Biophys. Acta, 319 (1973) 11.
4 T.P. Waalkes, C.W. Gehrke, W.A. Bleyer and H.R. Zumwalt, Cancer, 36 (1975) 390.
5 C.W. Gehrke, K.C. Kuo, G.E. Davis, R.D. Suits, T.P. Waalkes and E. Borek, J. Chromatogr., 150 (1978) 455.
6 M. Uziel, C.H. Koh and W.E. Cohn, Anal. Biochem., 25 (1968) 77.
7 R.A. Hartwick and P.R. Brown, J. Chromatogr., 143 (1977) 383.
8 R.A. Hartwick, A. Jeffries, A.M. Krstulovic and P.R. Brown, J. Chromatogr., 16 (1978) 427.
9 P.R. Brown, A.M. Krstulovic and R.A. Hartwick, in M.M. Muller and E. Kaiser (Editors), Purine Metabolism in Man, Plenum Press, New York, 1977.
10 H. Hahn, Plant Cell Physiol., 17 (1976) 1053.
11 F.S. Anderson and R. Murphy, J. Chromatogr., 121 (1976) 251.
12 D.B. Lakings, T.P. Waalkes and J.E. Mrochek, J. Chromatogr., 116 (1976) 83.
13 A.M. Krstulovic, P.R. Brown and D.M. Rosie, Anal. Chem., 49 (1977) 2237.
14 Y.M. Rustum, Anal. Biochem., 90 (1978) 289.
15 M. Uziel and C. Koh, J. Chromatogr., 59 (1971) 188.
16 C.W. Gehrke, K.C. Kuo, T.P. Waalkes and E. Borek, Cancer Res., 39 (1979) 1150.
17 J.E. Mrochek, S.R. Donsmore and T.P. Waalkes, J. Nat. Cancer Inst., 53 (1974) 1553.
18 K.B. Woo, T.P. Waalkes, D.L. Ahmann, D.C. Tormey, C.W. Gehrke and V.T. Oliverio, Cancer, 41 (1978) 1685.
19 P.F. Ma and J.R. Fisher, Biochim. Biophys. Acta, 159 (1968) 153.
20 M. Friedkin and H. Kalckar, in P.D. Boyer, H. Lardy and K. Myrback (Editors), The Enzymes, Vol. 5, Academic Press, New York, 1961, p. 237.
21 V.D. Hoagland, Jr. and J.R. Fisher, J. Biol. Chem., 242 (1967) 4341.
22 H.J. Breter and R.K. Zahn, Anal. Biochem., 54 (1973) 316.
23 H. Ratech, G.J. Thorbecke and R. Hirschhorn, in preparation.
24 J.F. Kuttesch, F.C. Schmalstieg and J.A. Nelson, J. Liquid Chromatogr., 1 (1978) 97.