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Note

Identification of fluorescent derivatives of adenosylmethionine and related analogues with high-pressure liquid chromatography

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AdoMet-dependent* transmethylation systems demonstrate product inhibition with AdoHcy[1] which suggests a regulatory role in cellular processes for this compound and its related analogues[1–3].

Most analytical procedures[4,5] for the identification of this class of metabolites, although accurate, are time-consuming. Several recent methods[2,6,7] have overcome this disadvantage.

Biochemical studies of nucleosides and nucleotides have been greatly facilitated by the use of fluorescent derivatives. One such approach involves the reaction of chloroacetaldehyde with Ade and Cyt to form their corresponding ε analogues[8,9]. The property of high fluorescence of εAde compounds (as opposed to εCyt derivatives) in aqueous media[8] is exploited in the work reported here.

In this paper, I describe a facile method in which HPLC is used for the separation, identification and quantitation of fluorescent derivatives of AdoMet and several analogues which contain Ade; I also demonstrate its applicability for measurement of enzyme activities.

EXPERIMENTAL

Materials

Chloroacetaldehyde (30% in water) was obtained from Columbia Organic Chemical Co. (Columbia, S.C., U.S.A.); Aminex A-6 from Bio-Rad Labs. (Richmond, Calif., U.S.A.); Ade from Calbiochem (Los Angeles, Calif., U.S.A.); Ado from P-L Biochemicals (Milwaukee, Wisc., U.S.A.); AdoMet and AdoHcy from Sigma (St. Louis, Mo., U.S.A.); and AdoSMe was a gift of Dr. J. Duerre, University of North Dakota School of Medicine.

HPLC

A 0.6 × 25 cm glass column (Laboratory Data Control, Model LC-6M-13)

* The abbreviations used are: HPLC = high-pressure liquid chromatography; AdoMet = adenosylmethionine; AdoHcy = adenosylhomocysteine; AdoSMe = methylthioadenosine; Ado = adenosine; Ade = adenine; rTase = tRNA (uracil-5-)-methyltransferase; AdoHcyase = adenosylhomocysteine nucleosidase; ε = 1,N^6-etheno; Cyt = cytosine.

was packed with the cation exchanger Aminex A-6 as a wet slurry and maintained at a constant temperature of 48° with a circulating water-bath (Brinkmann, Model Lauda-RC3). Solvent (0.85 *M* ammonium acetate at pH 5.7 in 5% v/v ethanol)[10] was pumped through the column (Laboratory Data Control, Minipump No. 396) at 2.1 cm/min (175 p.s.i.g. inlet pressure), and samples were applied via an injector septum by use of a 100-μl HPLC microsyringe (Pierce, Model LC-210). The column eluent was monitored simultaneously by an absorbance detector at 254 nm (Altex Scientific, Inc., analytical UV detector Model 152) and by a fluorescence detector (American Instruments Co., Fluoro-monitor). The fluorometer was equipped with a germicidal mercury lamp, a primary filter (Corning, No. 7-54) that transmitted radiation in the range 250–400 nm and a secondary filter (Kodak, Wratten gelatin filter No. 4) that transmitted radiation above 450 nm. The chromatographic data were recorded on a dual-pen recorder (Linear Instruments Corp.).

ε Derivatives

Authentic ε derivatives of Ade, Ado, AdoSMe, AdoHcy and AdoMet were prepared by the method of Secrist *et al.*[11] from the parent compounds.

Enzyme preparations

A semipurified AdoHcyase and rTase were prepared from *Escherichia coli* through the hydroxyapatite step[10].

RESULTS AND DISCUSSION

Authentic ε derivatives of AdoMet and related compounds were prepared[11] and chromatographed under the prescribed HPLC conditions. The retention times, relative fluorescence and relative absorbance of these derivatives and their parent compounds are reported in Table I. A linear spectrophotometric response of the parent compounds was observed between 1 and 200 nmoles, whereas a linear fluorometric response of the ε analogues was observed between 0.1 and 2 nmole.

In each instance the ε derivative demonstrated an increase in retention time relative to the parent compound; this is a reflection of the increase in positive charge on the ε derivative.

After derivatization, the sensitivity for detection of the various compounds increased by a factor of 10–20. A further two-fold increase in sensitivity has been attained through careful attention to the control of background noise in the system (data not shown).

The usefulness of this analytical method for determining product formation during enzyme catalysis was tested as follows. Reaction mixtures of AdoHcyase and rTase assays[10] were prepared in small glass test-tubes and allowed to incubate with the appropriate enzyme for 15 min at 30°. An equivalent volume of 2 *M* sodium acetate buffer (pH 4.5) was then added, along with 0.2 volumes of 30% chloroacetaldehyde. After the contents were thoroughly mixed, the test-tubes were sealed with Parafilm and incubated in a water-bath at 60° for 60 min. The tubes were then centrifuged in a desk-top centrifuge, and an aliquot of the centrifugate was injected onto the HPLC column. The fluorometric profile of each run was analyzed, and the amount of AdoHcy as εAdoHcy (0.030 nmoles) produced in the rTase assay and the

TABLE I

HPLC PROPERTIES OF AdoMet AND SOME RELATED METABOLITES AND THEIR ε DERIVATIVES

Compound	*Retention time (min)*	*Relative absorbance (units/nmole)* *	*Relative fluorescence (units/nmole)* *	*Minimum amount detectable (nmoles)* **
Ado	15	14.3		0.14
AdoHcy	19	8.8		0.23
Ade	30	11.7		0.17
AdoSMe	40	5.5		0.36
AdoMet	112	2.0		1.00
εAdo	26		341.4	0.006
εAdoHcy	36		171.0	0.012
εAde	48		146.4	0.014
εAdoSMe	73		123.3	0.016
εAdoMet	236		36.0	0.056

* Data were averaged from ten separate runs over the range of concentrations at which linear spectrophotometric and fluorometric responses were observed for each compound (see text). During a run, the recorder response (0–100 units) was measured at the retention time for each compound, and the units obtained were then normalized to the absorbance detector set at an optical density range of 0.08 and the fluorometer at a sensitivity setting of 10. The relative absorbance and fluorescence values represent the units one would obtain at the normalized settings for 1 nmole of compound.

** The quantity of compound which would give a recorder response of two units under the conditions specified above.

amount of Ade as εAde (0.055 nmoles) produced in the AdoHcyase assay were calculated from data collected with authentic ε compounds (Table I). These results were found to agree within $\pm$ 2% with data obtained separately[10] by the use of radioisotopes in the rTase assay and spectrophotometric measurements in the AdoHcyase assay.

It should be noted that the conditions for chemical modification have been optimized for the derivatization of Ade-containing compounds[8,9,11], and any weak fluorescence due to the contamination of εCyt-containing material would be cut off by the secondary filter. Furthermore, no correction is required in the fluorometric data for the reagent, and ,since only one solvent system is used, no regeneration of the column is necessary between runs.

The advantages of the method described here are:

(1) The intensity of fluorescence of the ε derivatives increases the sensitivity of detection of the compounds reported here by a factor of 10–20.

(2) The method is highly selective for Ade-containing compounds, which makes it useful for studies of AdoMet and its related analogues. This can be particularly important in studies of enzymic reactions involving these compounds in which one of them (product) is usually present in an extremely small amount relative to another (substrate).

(3) The method is conventional; because it uses published analytical procedures and existing HPLC instrumentation, it promises wide application.

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