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MICROASSAY FOR N-ACETYLTRANSFERASE ACTIVITY USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

A rapid, sensitive procedure has been developed for determination of N-acetyltransferase activity against octopamine, dopamine and 5-hydroxytryptamine. The assay, which is performed in a volume of 10 μl, is based upon the separation and detection of monoamine substrates and their N-acetylated derivatives using high-performance liquid chromatography with electrochemical detection. The method has been used to measure N-acetyltransferase activity against octopamine, dopamine and 5-hydroxytryptamine in the cerebral ganglion of the American cockroach, *Periplaneta americana* and to study bi-substrate kinetics of the enzyme.

INTRODUCTION

N-Acetyltransferase (NAT) (acetyl-coenzyme A:arylamine N-acetyltransferase, EC 2.3.1.5) acetylates the amino moiety of arylalkylamines using acetyl-coenzyme A (acetyl-CoA) as the acetyl donor. In mammalian pineal gland, NAT is a neurally induced enzyme that catalyzes the production of N-acetyl-5-hydroxytryptamine (N-ac-5-HT) which is the immediate precursor to melatonin [1]. The importance of melatonin as a putative circadian hormone has generated considerable interest in NAT as a site of neural activation and control.

The first routine NAT assay involved use of ^{14}C-labelled 5-hydroxytryptamine (5-HT) as substrate with subsequent isolation of the radiolabelled product by thin-layer chromatography [2]. This assay has been modified over the years with the TLC separation replaced by various organic solvent extractions (see ref. 3 for review) but no alternative methods have been proposed.

In addition to its role in melatonin formation, NAT is the major enzyme in-

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volved in inactivation of monoaminergic neurotransmitters in insects and, thus, fulfills the function of monoamine oxidase in vertebrates. Of particular interest is the N-acetylation of *p*-octopamine (OA), a primary sympathetomimetic transmitter in insects, to N-acetyl-*p*-octopamine (N-acOA) which is not amenable to efficient solvent extraction. Thus the classic NAT assays cannot be used for determination of NAT activity against OA.

The current study describes a new high-performance liquid chromatographic (HPLC) procedure coupled with electrochemical detection (ED) to determine NAT activity in microvolumes. The procedure enables estimation of NAT activity against OA, dopamine (DA) and 5-HT.

EXPERIMENTAL

Experimental materials

Cerebral ganglia (brains) were obtained from adult male American cockroaches, *Periplaneta americana*, taken one to five months after imaginal ecdysis from a colony maintained in this laboratory. OA, DA, 5-HT, N-acetyldopamine (N-acDA), N-ac-5-HT, tryptamine, ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT or Cleland's reagent) and acetyl-CoA were purchased from Sigma (St. Louis, MO, U.S.A.). HPLC-grade acetonitrile and methanol were obtained from Caledon (Ontario, Canada), 1-octanesulfonate (sodium salt) (SOSA) from Regis (Morton Grove, IL, U.S.A.) and the Bio-Rad protein assay kit from Bio-Rad Labs. (Toronto, Canada). N-acOA was generously donated by Dr. J.S. Kennedy (NIMH, Rockville, MD, U.S.A.). All other chemicals were Baker analytical grade from Canlab (Toronto, Canada).

N-Acetyltransferase extracts

NAT activity was assayed in tissue homogenates. Individual brains were dissected, blotted dry and placed in 100 μl ice cold 0.5 *M* potassium chloride and 1.3 m*M* DTT. Brains were homogenized either by sonication or glass–PTFE pestle. Sonication was achieved using a Branson Model 200 sonifier cell disruptor (Canlab) fitted with a micro-tip. The tissue was pulsed two times at 50% duty with 20 W output power. Pestle homogenisation was done with ten strokes of a motor-driven 1.0-ml conical glass–PTFE tissue grinder (Mandel Scientific, Rockwood, Canada). The motor drive (Canlab) was operated at approximately 300 rpm. The homogenates were then centrifuged at 40 000 *g* for 20 min at 4 °C. Both methods of homogenisation produced similar results with 90% of the NAT activity associated with the supernatant.

Following centrifugation, samples were held on ice for a maximum of 30 min prior to assay. A 4-μl volume of the resulting supernatant was assayed for NAT activity and protein content. Protein was determined using the microassay procedure of the Bio-Rad protein assay kit with bovine γ-globulin as the protein standard.

N-Acetyltransferase microassay

The incubation medium employed for the NAT assay was similar to that described by Hayashi et al. [4]; however, in order to reduce the cost of acetyl-CoA,

a microassay was developed with a final assay volume of 10 μl. The dispensing of microvolumes was facilitated by fitting a 100-μl gas-tight Hamilton syringe with a repeating dispenser (Canlab) and a removable needle which was burr-free and cut squarely at its end (Hamilton point style 3). Each 'click' of the repeating dispenser results in the discharge of 1/50 of the total volume. Thus, the syringe pipettor accurately dispensed 2-μl aliquots into V-shaped wells of a 96-well polystyrene Titertek® microplate (Flow Labs., Toronto, Canada) so that a droplet formed in the 'V' at the bottom of the well.

Temperature was regulated by floating the Titertek plate in a water bath. A 10-min pre-incubation at 30°C comprised 2 μl sodium phosphate buffer, 2 μl water or water with potential inhibitor and 4 μl tissue extract. The reaction was started with the addition of 2 μl of a solution containing the monoamine substrate and acetyl-CoA. The final assay cocktail comprised 200 m*M* potassium chloride, 20 m*M* sodium phosphate buffer (pH 7.0), 0.52 m*M* DTT, 1.0 m*M* monoamine substrate and 2.5 m*M* acetyl-CoA.

The Titertek plates were covered with Parafilm® (Canlab) for the duration of the incubations. The reaction was terminated with the addition of 100 μl of ice cold 0.1 *M* perchloric acid. This acidified mixture was allowed to stand on ice for 10 min and 15 μl were diluted further with 200 μl of 0.1 *M* perchloric acid. A 5-μl aliquot of this dilution was injected directly onto the HPLC system for quantitation of the N-acetylated product and/or monoamine substrate by ED.

HPLC analysis

Chromatographic separations were performed as previously described [5] at ambient temperature on an Ultrasphere I.P. C_{18} column (250 mm×4.6 mm I.D.,

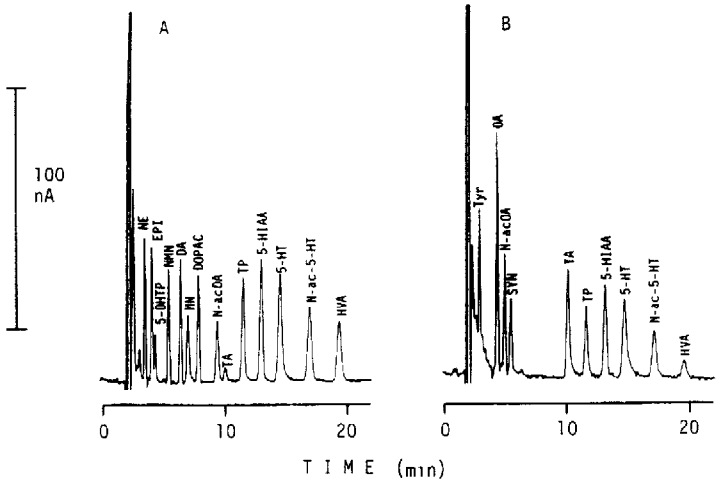


Fig. 1 Chromatograms of standards detected with detector 1 at +0.5 V (A) and detector 2 at +0.75 V (B) (see ref. 5) Standards: 500 pg norepinephrine (NE), epinephrine (EPI), normetanephrine (NMN), dopamine (DA), metanephrine (MN), 3,4-dihydroxyphenylacetic acid (DOPAC), N-acetyldopamine (N-acDA), tyramine (TA), tryptophan (TP), 5-hydroxyindoleacetic acid (5-HIAA), 5-hydroxytryptamine (5-HT), N-acetyl-5-hydroxytryptamine (N-ac-5-HT), homovanilic acid (HVA), tyrosine (Tyr), *p*-octopamine (OA), N-acetyl-*p*-octopamine (N-acOA); 200 pg *p*-synephrine (SYN); 100 pg 5-hydroxytryptophan (5-OHTP).

TABLE I

N-ACETYLATION OF *p*-OCTOPAMINE, DOPAMINE AND 5-HYDROXYTRYPTAMINE BY N-ACETYLTRANSFERASE FROM BRAIN OF *PERIPLANETA AMERICANA*

Values indicate the mean ± standard deviation for five determinations. All amine substrates were 1.0 m*M*.

Substrate	N-Acetylated amine (nmol per mg protein per min)		
	OA	DA	5-HT
OA	5.30 ± 0.29	-	-
DA	-	9.94 ± 0.44	-
DA + OA	2.37 ± 0.11	7.33 ± 0.56	-
5-HT	-	-	3.58 ± 0.18
5-HT + OA	4.20 ± 0.13	-	2.26 ± 0.13

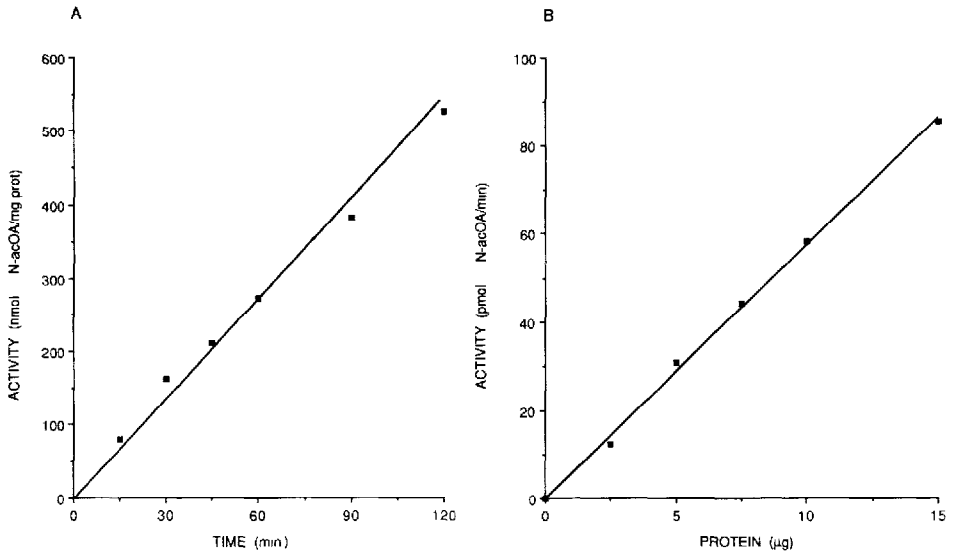
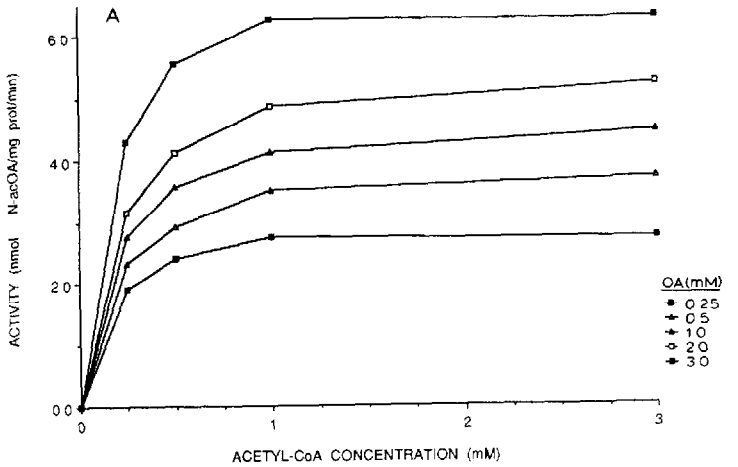
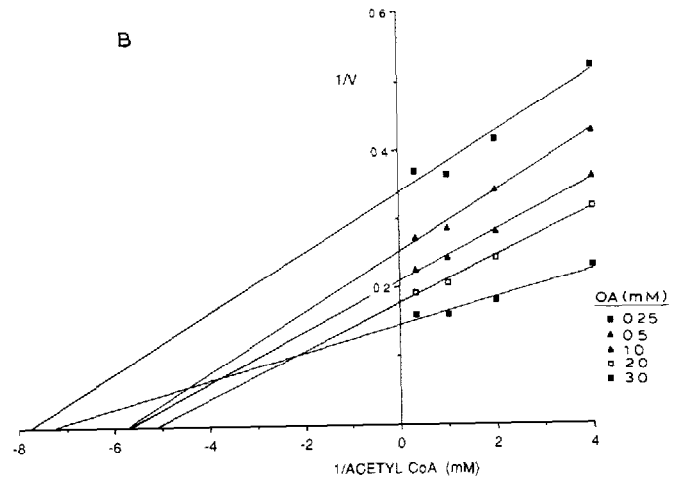
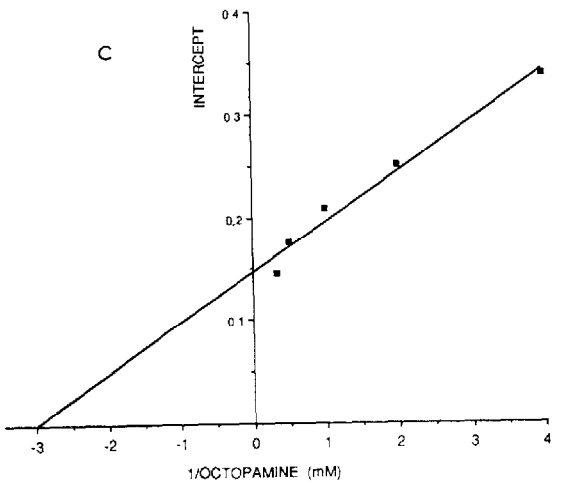


Fig. 2. (A) Time course (sample contained 6 7 µg protein) and (B) linearity with respect to protein (incubation time was 60 min) of N-acetyltransferase assays for crude enzyme preparation from cockroach brain. Each point represents the mean of four determinations. Standard errors of the mean are less than 5% of the means and r^2 values for each curve equal 0 99.

Fig. 3. (A) NAT activity from cockroach brain preparation at different concentrations of both substrates Acetyl-CoA concentrations were varied at five fixed concentrations of OA. Each point represents the mean of eight determinations. Standard errors of the means are less than 5% of the means. (B) Lineweaver–Burke plots of (A). Each curve represents a least-squares long fit using the FORTRAN program, HYPERL, by Cleland [8]. (C) Secondary plot of the ordinate intercepts of (B) versus the inverse of the respective fixed OA concentrations. (K_M for OA = 0.33 m*M*; V_{max} = 6.8 nmol N-acOA formed per mg protein per min). Standard errors of the intercepts are less than 5% and the r^2 value for the curve is 0.99.


A
ACTIVITY (nmol N-acOA/mg prot/mn)
6 0
4 0
2 0
0 0
0
1
2
3
ACETYL-CoA CONCENTRATION (mM)
OA(mM)
0 25
0 5
1 0
2 0
3 0


B
1/V
0 6
0 4
0 2
-8
-6
-4
-2
0
2
4
1/ACETYL CoA (mM)
OA (mM)
0 25
0 5
1 0
2 0
3 0


C
INTERCEPT
0 4
0 3
0,2
0 1
-3
-2
-1
0
1
2
3
4
1/OCTOPAMINE (mM)

5-μm spherical particles). A Brownlee C_{18} guard column (15 mm$\times$3.2 mm I.D., 7.2-μm spherical particles) and a 0.5-μm pre-column filter were installed between the injector (WISP 710A, Waters, Mississauga, Canada) and the analytical column. This afforded sufficient protection so that direct injection of the diluted assay cocktail had no apparent deleterious effects on the analytical column even after 5000 injections.

The mobile phase comprised 75 m*M* monobasic sodium phosphate, 1.0 μM EDTA, 0.35 m*M* SOSA, 11% methanol and 4% acetonitrile. Final apparent pH was adjusted to 4.0 with concentrated phosphoric acid. Prior to addition of the organic modifiers, the buffer was filtered through a 0.22-μm Millipore filter (Type GS). The mobile phase was continuously degassed with a stream of helium and delivered at 1.0 ml/min using a Spectra-Physics Model 740B pump equipped with two pulse dampeners (Waters).

The detector consisted of an ESA 5100A coulometric detector (ESA, Bedford, MA, U.S.A.) with a Model 5010 dual-electrode detector cell. Detection of OA and N-acOA was achieved at a potential of +0.75 V while detection of DA, 5-HT and their N-acetylated metabolites was accomplished at +0.50 V.

RESULTS AND DISCUSSION

The HPLC apparatus and chromatographic conditions described provide a clean chromatographic separation of OA, DA, 5-HT and their respective N-acetylated derivatives. Typical separations are shown in Fig. 1 which is taken from an earlier publication [5]. The order of elution is OA, N-acOA, DA, N-acDA, 5-HT and N-ac-5-HT and the detection limit for each compound is estimated conservatively at less than 100 pg. Thus measurement of the products of NAT-catalyzed reactions can be based on quantitation of the substrate consumed and/or metabolite produced with high sensitivity. This permits NAT activity in crude enzyme preparations to be measured with as little as 2.5 μg protein.

A typical HPLC analysis for N-acOA, N-acDA and N-ac-5-HT requires 6, 10 and 20 min, respectively, so that an HPLC system equipped with an autoinjector can analyse 70–250 samples (depending on the monoamine substrate) in a 24-h period with the mobile phase and columns as described. The utility of using a chromatographic system which resolves OA, DA, 5-HT and their derivatives in a single analysis is illustrated in Table I. The capacity to estimate enzyme activities using different substrates, and combinations of substrates, enables determination of substrate preferences and specificity.

However, for kinetic studies, it is desirable to keep the HPLC analysis time to a minimum. Thus, the use of shorter HPLC columns or higher solvent concentrations in the mobile phase is more suitable when DA or 5-HT are used as substrate. When high concentrations of substrate are used in the enzyme reaction, the substrate peak may begin to crowd and, thus, interfere with detection of the product which elutes after the substrate. Under these conditions, the order of elution of the monoamines with respect to their N-acetylated derivatives can be reversed by increasing the concentration of ion-pair reagent (SOSA) in the mobile phase. This results in a concomitant increase in retention of the monoamines and decrease in retention of the N-acetylated compound.

The enzymatic reaction is linear with time for at least 120 min (Fig. 2A) with the tissue preparation used in this study. It is also linear with increasing protein (enzyme) concentration (Fig. 2B). These attributes, coupled with the ability to use a wide range of substrate concentrations due to the sensitivity, flexibility and capacity of the HPLC analysis, demonstrate that the assay is suitable for bi-substrate kinetic studies. For example, Fig. 3 illustrates the three plots required to determine the Michaelis constant (K_M) for OA and V_{max} of the reaction according to the methods of Cleland [6] and Kontro and Oja [7]. The apparent K_M for OA is 0.33 mM and is obtained by calculating the negative inverse of the abscissa intercept of Fig. 3C. The ordinate intercept of Fig. 3C represents $1/V$, therefore, V_{max} is the inverse of the intercept (6.8 nmol N-acOA formed per mg protein per min).

The protocol described is suitable for routine enzyme analysis and reduces greatly the extent of post-assay sample manipulation from those required in the classic assay procedure. The microassay also eliminates the use of radiolabelled substrates and reduces the cost per assay of acetyl-CoA.

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