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Note

Determination of N-acetyldopamine by liquid chromatography with electrochemical detection

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High-performance liquid chromatography with electrochemical detection has proved very useful for the determination of catecholamines in animal tissues [1]. Suitable columns and mobile phases can be found that allow catecholamines to be assayed with detection limits at the picogram level. We report here an assay for N-acetyldopamine using liquid chromatography with electrochemical detection. N-Acetyldopamine is a substance long known to be present in insects and to be involved in biosynthesis of the cuticle. In recent years evidence has accumulated that indicates that N-acetyldopamine might also occur in insect nervous tissues [2—5], where it presumably has some function related to neural activity. Very recently an enzymatic—radiochemical assay for N-acetyldopamine has become available [6], but it has not been applied to insect or other arthropod tissues. Using the liquid chromatographic method with electrochemical detection described here we have obtained evidence that N-acetyldopamine occurs widely in insect nervous tissues and that smaller amounts of it occur in other non-cuticular insect tissues, as reported elsewhere [7]. Quantitative methods for the determination of N-acetyldopamine may be of clinical interest in view of the demonstrations of N-acetyldopamine in patients with phaeochromocytoma and neuroblastoma [8,9].

EXPERIMENTAL

Materials

The catecholamines and related compounds were purchased from Sigma (St. Louis, MO, U.S.A.); all other chemicals were of analytical-reagent grade. Alumina was purchased from Bioanalytical Systems (West Lafayette, IN,

U.S.A.) and used without further purification. N-Acetylnorepinephrine was synthesized by the method of Wolfe and Thorn [10]. Except for the mobile phase and physiological saline, all solutions were prepared in water redistilled in glass from alkaline permanganate (0.05 *M* NaOH and 0.05 *M* $KMnO_4$).

Cockroaches (*Periplaneta americana* L.) were reared in the laboratory and fed a diet of dog pellets ad libitum. Only adult males were used. Mice were adult males of the Cox (Swiss) strain obtained from Laboratory Supply (Indianapolis, IN, U.S.A.).

Apparatus

The chromatographic system consisted of a Milton-Roy mini-pump (Milton-Roy, Riviera Beach, FL, U.S.A.), pulse damper (Bioanalytical Systems, West Lafayette, IN, U.S.A.), a Rheodyne Model 70-10 injection valve with a 20-μl sample loop (Rheodyne, Berkeley, CA, U.S.A.) and a Partisil 10 ODS II analytical reversed-phase column (25 × 0.46 cm I.D.) (Whatman, Clifton, NJ, U.S.A.) fitted with a 6 × 0.5 cm I.D. pre-column packed with Bondapak C_{18} Corasil (Waters Assoc., Milford, MA, U.S.A.). The electrochemical detector consisted of a carbon paste, wax-impregnated graphite—oil electrode (CP-W) and LC-2A potentiostat from Bioanalytical Systems. The working electrode was set at +0.72 V relative to an Ag/AgCl reference electrode. Peaks were recorded with an Omniscribe recorder (Houston Instruments, Austin, TX, U.S.A.). All chromatograms were obtained at ambient temperature (20—22°C).

Procedures

The insect tissues were dissected out under ice-cold saline (160 m*M* NaCl, 8 m*M* KCl, 1.8 m*M* $CaCl_2$, 0.2 m*M* Na_2HPO_4, 1.8 m*M* NaH_2PO_4, pH 7.2 [11]) saturated with phenylthiourea to inhibit phenoloxidase activity. Once the tissue had been freed of extraneous tissues, including tracheae as far as possible, excess of saline was removed by absorption with a piece of filter-paper and the tissue was frozen on a porcelain plate kept on dry-ice. The collected tissues were subsequently weighed to the nearest 0.1 mg on a Cahn 7500 electrobalance, transferred to an all-glass homogenizer (200-μl capacity; Radnoti Glass Technology, Arcadia, CA, U.S.A.) and homogenized in 100 μl of 0.1 *M* $HClO_4$ containing 0.0004 *M* sodium bisulfite. Homogenization was continued until fragments or strings of intact tissue were no longer visible. The homogenates were centrifuged for 10 min at 500 *g*. Duplicate 40-μl aliquots of each supernatant were pipetted into 1.5-ml Eppendorf polypropylene micro test-tubes, 10 pmol of N-acetyldopamine in 0.1 *M* $HClO_4$ were added to one of these aliquots and the volume of this and the duplicate sample were adjusted to 100 μl with 0.1 *M* $HClO_4$. By comparing peak heights with and without added N-acetyldopamine it was possible to calculate the recovery of endogenous N-acetyldopamine.

N-Acetyldopamine and the other catechol compounds were adsorbed on to alumina by adding to each tube 22.5 mg of a mixture of alumina and Na_2EDTA [12] (2:1, w/w) and 1.0 ml of 0.5 *M* Tris—HCl buffer (pH 8.6). The tubes were capped and shaken vigorously on a wrist-action shaker for 15 min. After brief centrifugation (2 min at 12,800 *g* in an Eppendorf 5412 centrifuge) to sediment the alumina, the supernatant was aspirated off and discarded. The alumina

was then washed once as follows: 1.0 ml of 0.0004 *M* sodium bisulfite was added, the tubes were shaken vigorously on the wrist-action shaker for 2 min and then centrifuged to sediment the alumina. The supernatant was next aspirated off and discarded. Care was taken to remove as much of the wash solution as possible during aspiration. This operation was facilitated by aspirating through a Pasteur pipette that had been drawn to a fine point.

N-Acetyldopamine was desorbed from the alumina by adding 100 μl of 1.0 *M* acetic acid containing 0.0004 *M* sodium bisulfite. The mixture was shaken for 15 min on the wrist-action shaker. After brief centrifugation to pellet the alumina, the supernatant containing N-acetyldopamine and other catechol compounds was collected and injected into the chromatograph. The mobile phase was 0.06 *M* sodium citrate, 0.04 *M* Na_2HPO_4 [13] and 0.0001 *M* Na_2-EDTA containing 20% (v/v) of methanol (final pH 3.6) and was pumped at a rate of 1 ml/min.

RESULTS AND DISCUSSION

Table I gives the retention times of catecholamines and other compounds that might interfere in the determination of N-acetyldopamine. The chromatogram shown in Fig. 1A shows that N-acetyldopamine is well separated from numerous catechol compounds or derivatives that might occur in animal tissues together with N-acetyldopamine. Fig. 1B shows the presence of an N-acetyldopamine peak in an extract of cerebral ganglion from the cockroach *P. americana*, the peak representing 1.3 pmol of N-acetyldopamine. After correction for recovery, the concentration of N-acetyldopamine in cerebral ganglion in the sample analysis shown was calculated to be 1.9 μg/g wet weight. The mean value for N-acetyldopamine in adult male *P. americana* cerebral ganglion was 2.09 ± 0.25 μg/g wet weight (n=25). The peaks near the solvent front in Fig. 1B are due to dopamine and several additional polar catechol compounds that are adsorbed on to alumina but not retained on the reversed-phase column. The only naturally occurring catechol compound tested that elutes near N-acetyldopamine under the chromatographic conditions described is 3,4-dihydroxyphenylacetic acid, but it elutes early enough not to interfere seriously with N-acetyldopamine determination. We have never been able to detect 3,4-dihydroxyphenylacetic acid in insect tissues but it is easily demon-

TABLE I

RETENTION TIMES OF CATECHOLAMINES AND RELATED COMPOUNDS

Conditions as described under *Procedures*

Compound	Retention time (min)	Compound	Retention time (min)
Norepinephrine	3.3	Metanephrine	4.2
Epinephrine	3.5	Epinine	4.3
3,4-Dihydroxynorephedrine	3.5	N-Acetylnorepinephrine	4.9
3,4-Dihydroxybenzylamine	3.6	3,4-Dihydroxyphenylalanine methyl ester	5.8
3,4-Dihydroxyphenylalanine	3.7	3,4-Dihydroxyphenylacetic acid	8.1
Normetanephrine	3.8	N-Acetyldopamine	10.0
Dopamine	4.0	N-Acetyl-5-hydroxytryptamine	18.4
Isoproterenol	4.2		

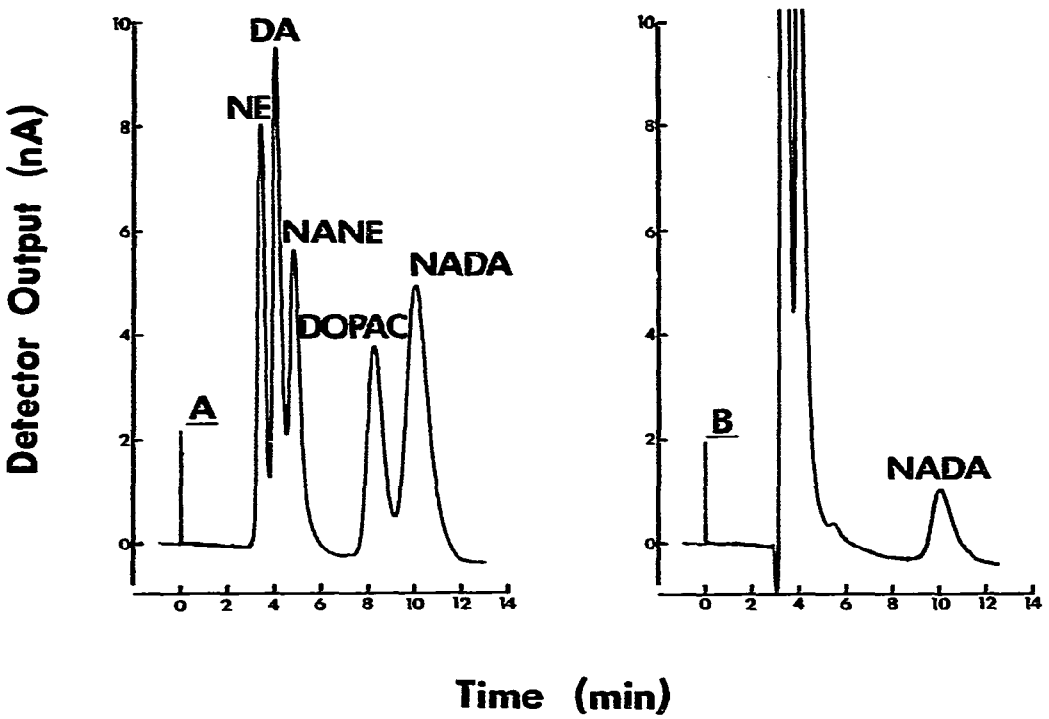

Fig. 1. High-performance liquid chromatograms showing electrochemical detector output after injecting (A) 5 pmol of N-acetyldopamine (NADA), norepinephrine (NE), dopamine (DA), N-acetylnorepinephrine (NANE) and 3,4-dihydroxyphenylalanine (DOPAC), and (B) extract of cerebral ganglion from an adult male *P. americana*.

strable and quantifiable in normal mouse brain using the present procedure. On the other hand, we have been unable to demonstrate the presence of N-acetyldopamine (< 0.02 μg/g) in whole brain extracts from normal adult Cox (Swiss) laboratory mice.

The detector responses to increasing amounts of N-acetyldopamine were linear over the range up to 20 pmol. Over the same range, the recovery from the alumina adsorption step was also linear, with a mean value of 51.0 ± 2.0%. Although alumina adsorption procedures for the isolation and assay of catecholamines frequently involve multiple washing steps after adsorption of the amines, we found that a single washing step eliminated any interfering substances. Further washes served only to reduce the recovery. Correlation coefficients determined by linear regression of the amount of N-acetyldopamine injected versus peak height were $r^2 = 0.99$ for authentic N-acetyldopamine injected directly into the chromatograph and $r^2 = 0.99$ for standard N-acetyldopamine carried through the alumina adsorption step.

When the concentration of methanol in the mobile phase was increased above 20%, N-acetyldopamine eluted faster but the life of the carbon paste electrode was markedly reduced. Decreasing the concentration of methanol below 20% increased the retention time of N-acetyldopamine and resulted in a marked broadening of the peak. The methanol concentration of 20% (v/v)

represents the best compromise between speed of elution, peak height, and convenience for analysis.

The selectivity and specificity of the method depends on the selectivity of the extraction of catechol compounds on to alumina, the retention of N-acetyldopamine as a moderately polar compound on the reversed-phase column and the ease of its oxidation in the electrochemical detector. The minimum amount of N-acetyldopamine detectable with our method was ca. 0.12 pmol or 25 pg. This amount of N-acetyldopamine produced a peak that was approximately twice as high as the baseline noise on the strip-chart recorder.

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