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DETERMINATION OF CATECHOLAMINES BY RADIOENZYMATIC ASSAY USING ION-PAIR LIQUID CHROMATOGRAPHY

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SUMMARY

A simple radioenzymatic method for the simultaneous determination of norepinephrine, epinephrine and dopamine in human serum was developed. Catecholamines were converted to normetanephrine (NMN), metanephrine (MN), 3-methoxytyramine (3-MT) and their 4-O-methyl isomers by catechol O-methyl transferase with S-adenosylmethionine-(^{3}H-methyl) ([^{3}H]SAM). After addition of unlabelled NMN, MN and 3-MT as carriers, the methyl derivatives were isolated from the reaction mixture by passing through a small boric acid gel column and separated from each other by ion-pair liquid chromatography. Interference by tailing of radioactivity of [^{3}H]SAM and its degradation products eluted earlier was minimized by the use of a precolumn and a branched flow path.

INTRODUCTION

A radioenzymatic assay of catecholamines has been developed by combining the selectivity of the methylation reaction of catechol O-methyl transferase (COMT) and the sensitivity of radioactivity determination. Generally tritium-labeled normetanephrine (NMN), metanephrine (MN) and 3-methoxytyramine (3-MT) produced in the enzyme reaction, are separated from unreacted S-adenosylmethionine-(^{3}H-methyl) ([^{3}H]SAM) and its degradation products by either solvent extraction [1,2] or precipitation with phosphotungstic acid [3] and isolated separately by thin-layer [1,2] or paper chromatography [4,5] followed by

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determination of the radioactivity. Recently, separation of 3-O-methyl derivatives was performed by high-performance liquid chromatography [6, 7]. However, in the procedures previously reported, formation of 4-O-methyl derivatives was not described, in spite of the possible formation of 4-O-methyl isomers during the enzyme reaction [8, 9].

In this paper we have used a boric acid gel column to adsorb unreacted [^{3}H]-SAM and [^{3}H]methylthioadenosine (MTA), demonstrated the formation of 4-O-methyl isomers and separated three pairs of methyl derivatives of catecholamines by high-performance liquid chromatography for the accurate determination of catecholamines in biological materials.

EXPERIMENTAL

Reagents

S-Adenosylmethionine-(^{3}H-methyl) (spec. act. 11.0 and 85.0 Ci/mmol), ^{14}C-dopamine·HBr (spec. act. 54.0 mCi/mmol) and Aquasol-II scintillation cocktail were obtained from New England Nuclear (Boston, MA, U.S.A.). COMT (2250 units/mg protein) purified from porcine liver was obtained from Sigma (St. Louis, MO, U.S.A.). A radioenzymatic assay kit of catecholamines, KAT-A-KIT™, which uses COMT from rat liver, was obtained from Upjohn (Kalamazoo, MI, U.S.A.). Boric acid gel was purchased from Aldrich (Milwaukee, WI, U.S.A.). 3,4-Dihydroxybenzylamine (DHBA)·HCl and vanillylamine·HCl were synthesized according to the method of Nelson [10]. All other chemicals were highest grade reagents.

Preparation of deproteinized serum

Blood collected by vein puncture was left for 15 min at 4°C and centrifuged to separate the serum. Three microliters of 5 *N* perchloric acid were added to 60 μl of serum and precipitated protein was removed by centrifugation at 10,000 *g* for 30 min. Supernatant (50 μl) was used for the enzyme reaction.

Preparation of a boric acid gel column

Boric acid gel was allowed to swell in 5% aqueous acetone overnight and activated by washing with 1 *N* HCl, water, 0.1 *N* NaOH and water successively and stored in water. Columns (17 mm × 3 mm I.D.) were prepared with fresh gel for each determination.

Enzyme reaction and sample preparation for chromatography

The buffer solution for the enzyme reaction was prepared to contain 2.5 *M* Tris, 50 m*M* EGTA, 400 m*M* $MgCl_2$ and 1.3 *M* mercaptoethanol. The pH was adjusted to 8.8 using HCl. Fifty microliters of a standard mixture of catecholamines or deproteinized serum were placed in 5-ml glass-stoppered centrifuge tubes cooled on ice. The buffer solution of enzyme reaction (10 μl), [^{3}H]SAM (1 μCi), porcine COMT (30 units) and DHBA (10 pg) were added and the total volume was adjusted to 100 μl with water. The solution was mixed with a vortex mixer for 5 sec and incubated for 45 min at 37°C. The reaction was stopped by the addition of 7 *N* NaOH (5 μl). Carrier solution (5 μl) containing 75 μg of NMN, MN and 3-MT was added. The solution was mixed with a vortex

mixer for 10 sec and centrifuged. Supernatant (100 μl) was applied to the boric acid gel column and O-methylated derivatives were eluted with 3 × 100 μl of 250 m*M* Tris—NaOH solution (pH 13.0). Eluate was collected in a tube containing 18% HCl (16 μl); a quarter of the eluate was subjected to ion-pair liquid chromatography.

Chromatography

A high-performance liquid chromatograph was assembled from a pump (Kyowa Seimitsu KHU-52H, Tokyo, Japan), a short column (10 cm × 4 mm I.D.), a 254-nm UV detector (Shimadzu UVD-2, Kyoto, Japan) and a fraction collector (Tokyo Rikakikai DC-180, Tokyo, Japan). Columns were packed with LiChrosorb RP-18, 10 μm, by the balanced density method. The mobile phase was acetonitrile—water (7:93, v/v) containing 0.5 g of sodium heptanesulfonate and 1.4 or 4.0 ml of acetic acid per liter and was adjusted to pH 4.0 with 15% NaOH [11]. The flow-rate was 1.0 ml/min.

Branched flow path

The flow system (Fig. 1) was essentially the same as the one devised for large-volume sample injection in gas chromatography [12]. After sample injection, the outlet of the precolumn was connected directly to the detector and elution was monitored by UV absorption. After elution of the peak (shown by an arrow in Fig. 2A) originating from mercaptoethanol in the enzyme reaction mixture, the main column was connected as in Fig. 1. Essentially all the radioactivity of degradation products and [^{3}H]SAM was eluted from the precolumn before this stage and thus contamination of the main column was avoided. Radioactivity was determined on every 0.5-ml fraction with 5 ml Aquasol-II using an Aloka liquid scintillation spectrograph (Aloka LSC-903, Tokyo, Japan).

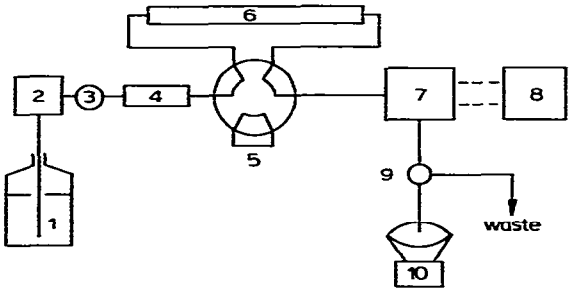

Fig. 1. Schematic diagram of the flow system. 1 = Eluent reservoir; 2 = pump; 3 = line sample injector; 4 = precolumn; 5 = six-way valve; 6 = analytical column; 7 = detector; 8 = recorder; 9 = three-way valve; 10 = fraction collector.

RESULTS

Chromatographic separation of methyl derivatives

The chromatogram of the reaction mixture of dopamine (DA) displayed four peaks by radioactivity determination [Fig. 2B]. The peak appearing at the front was a mixture of [^{3}H]SAM and its degradation products. Degradation of SAM

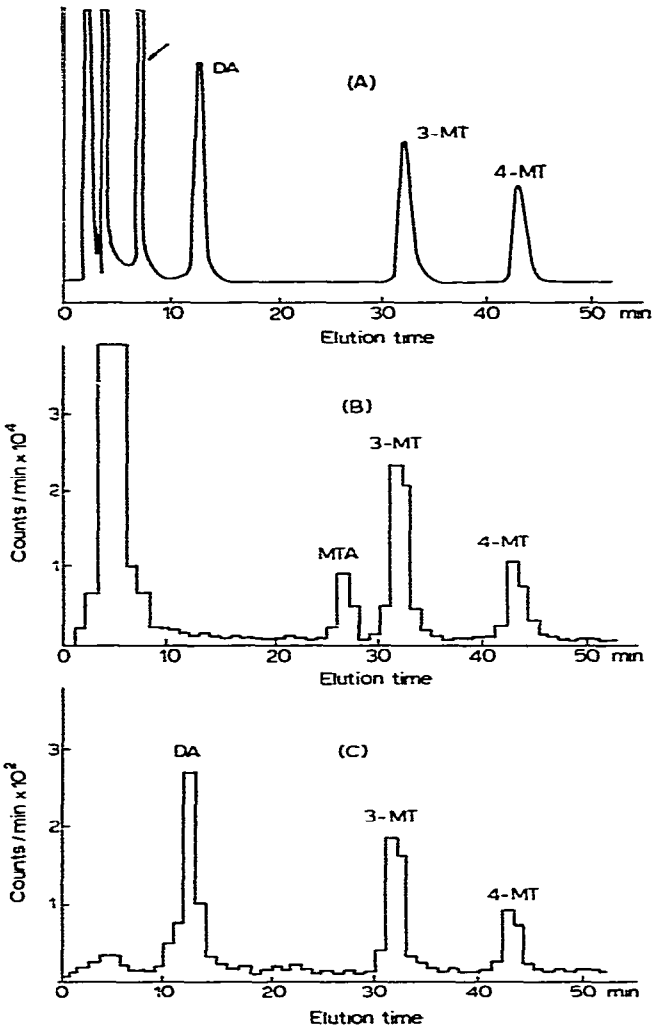

Fig. 2. Chromatogram of dopamine and its O-methylated derivatives using UV and radioactivity detection (A, absorbance at 254 nm; B, ^{3}H radioactivity; C, ^{14}C radioactivity). Sample was a quarter of a 100-μl reaction mixture of [^{14}C]dopamine (15 ng, 4 μCi), porcine COMT (30 units) and [^{3}H]SAM (1 μCi, spec. act. 11.0 Ci/mmol). DA, 3-MT and 4-MT (75 μg each) were added as carriers after enzyme reaction. Column was LiChrosorb RP-18, 10 μm, 250 × 4 mm I.D. Eluent was acetonitrile—water (7 : 93, v/v) containing 0.5 g of sodium heptanesulfonate and 4.0 ml of acetic acid (adjusted to pH 4.4 with NaOH). Flow-rate was 1.0 ml/min; temperature, ambient.

to MTA was also observed. Two peaks appeared from the enzyme reaction of dopamine and their elution times corresponded to those of authentic 3-MT and 4-MT (Fig. 2A and B). The peaks of dopamine derivatives were further examined by methylating [^{14}C]dopamine under the same conditions (Fig. 2C). The ratio of ^{14}C to ^{3}H in each peak was identical and essentially the same as the ratio of specific activity of [^{14}C]dopamine and [^{3}H]SAM. The relative quantity of 3-MT to 4-MT was not significantly changed when rat liver COMT in the KAT-A-KIT was used in place of porcine liver COMT.

The enzymatic methylation of norepinephrine or epinephrine also yielded two peaks. The peaks eluted earlier had retention times corresponding to authentic NMN and MN. Peaks eluting later were assumed to be 4-O-methyl derivatives. In these chromatographic conditions, six peaks from three catecholamines and MTA were resolved and no other distinct peak was observed except for [^{3}H]SAM and its degradation products which eluted at the front. However, a high level of radioactivity, 250—300 cpm, was observed between peaks (Fig. 3).

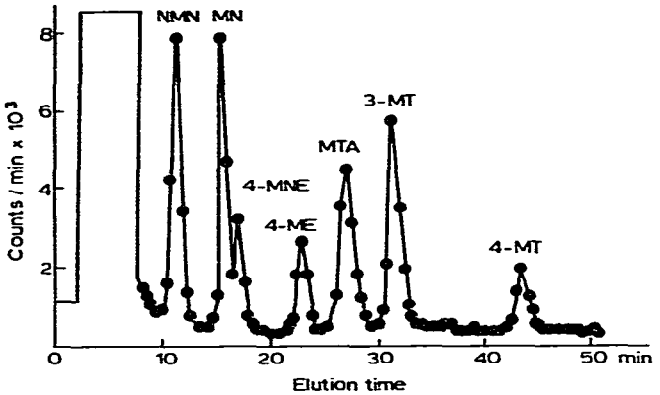

Fig. 3. Chromatogram of O-methylated catecholamines. Sample was a quarter of a 100-μl reaction mixture of catecholamines (4 ng each). Other conditions as in Fig. 2.

Boric acid gel treatment and branched flow with a precolumn

Extraction of the methyl derivatives with a mixed solvent of isoamyl alcohol and toluene is a usual procedure to separate them from [^{3}H]SAM and its degradation products. In place of solvent extraction, a combination of two simple procedures was examined in the present investigation. A gel which has dihydroxyboronyl groups chemically bonded on to polystyrene—dinivylbenzene copolymer is available and has been used to adsorb nucleosides [13], saccharides [14] and catecholamines [15]. Compounds with vicinal diol groups in the reaction mixture, such as SAM and MTA, were expected to be adsorbed by the boric acid gel from alkaline solution.

Elution of the methyl derivatives from the boric acid gel column was examined (Table I). The methyl derivatives were quantitatively eluted with 0.25 *M* Tris—NaOH solution (pH 13), while almost all of the SAM and MTA was retained by the column. However, an excessive amount of radioactivity from degradation products, appearing at the front of the chromatogram in Fig. 3, was not adsorbed by the boric acid gel.

Because the radioactivity of baseline fractions decreased as elution was continued and reached the level of natural background after about 2 h, small quantities of radioactive compounds seemed to be adsorbed strongly on the column and eluted gradually, resulting in prolonged tailing. To minimize contamination of the column with radioactivity from the degradation products, the flow system in Fig. 1 was used.

By employing the boric acid gel treatment and branched flow system, radioactivity of the baseline fractions was reduced to ca. 50 cpm, roughly twice the natural background.

TABLE I

EFFECT OF pH OF ELUENT ON RECOVERY (%) OF 3-O-METHYL CATECHOLAMINES, SAM AND MTA FROM BORIC ACID GEL COLUMN

Sample solution was 3-O-methyl catecholamines (75 μg) or SAM and MTA (2 μg) in the buffer solution (100 μl). The sample solution was made alkaline and passed through a boric acid gel column (17 × 3 mm I.D.). Elution was carried out with 3 × 100 μl of 250 m*M* Tris—NaOH solution of various pH values.

Compound	pH of eluent				
	10.0	11.0	12.0	13.0	13.7
NMN	78	95	100	100	85
MN	52	76	87	100	85
3-MT	18	35	41	98	88
SAM				5	52
MTA				5	40

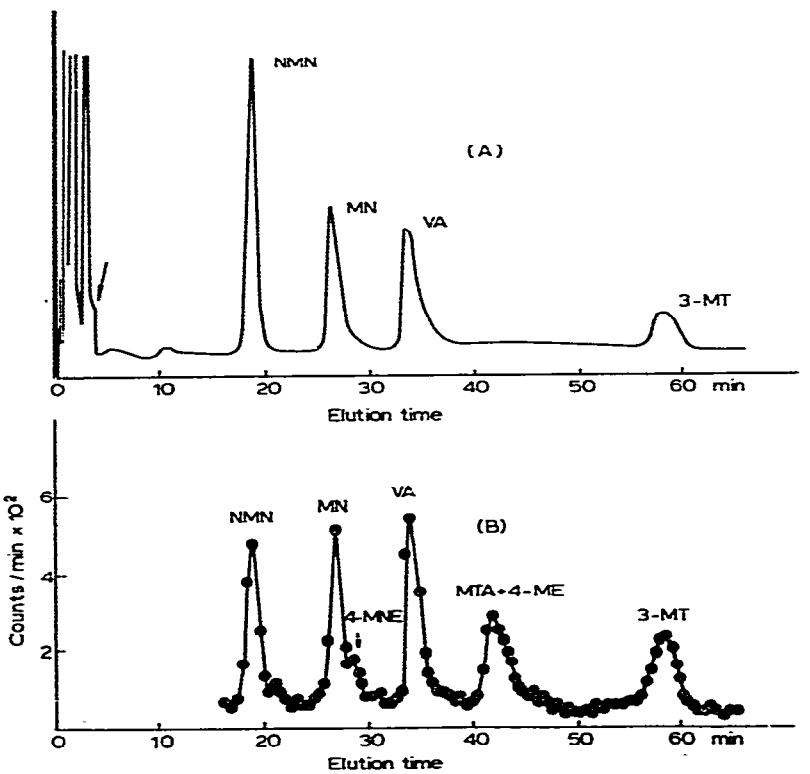

Fig. 4. Chromatograms of O-methylated catecholamines and vanillylamine using the branched flow system (A, absorbance at 254 nm; B, ^3H radioactivity). Sample was a quarter of a reaction mixture of catecholamines (15 pg each), porcine COMT (30 units) and [^3H]-SAM (1 μCi, spec. act. 85.0 Ci/mmol) with carriers after boric acid gel treatment. Precolumn was LiChrosorb RP-18, 10 μm, 100 × 4 mm I.D. Main column was LiChrosorb RP-18, 10 μm, 250 × 4 mm I.D. Eluent was acetonitrile—water (7:93, v/v) containing 0.5 g of sodium heptanesulfonate and 1.4 ml of acetic acid (pH adjusted to 4.4 with NaOH) per liter. Flow-rate was 1.0 ml/min; temperature, ambient. Main column is connected at the time shown by the arrow (chromatogram A).

A chromatogram of a standard mixture of the three catecholamines was obtained using the above procedure (Fig. 4). The amounts of the methyl derivatives injected corresponded to ca. 4 pg of catecholamines in the enzyme reaction mixture. The time required for elution of the methyl derivatives was increased by the use of the precolumn and reduction of the acetic acid concentration in the eluent. 4-Methylnorepinephrine (4-MNE) was eluted as a shoulder of the MN peak and a peak of 4-methylepinephrine (4-ME) overlapped that of MTA leaked from the boric acid gel column.

DHBA was also methylated by COMT and was added to the reaction mixture to detect accidental or unexpected failure of the enzyme reaction. Under the reaction conditions used, approximately 70% of DHBA was converted to vanillylamine, while no significant amounts of isovanillylamine, the 4-methyl derivative of DHBA, which should elute between MTA and 3-MT, were formed.

Determination of catecholamines in serum

Catecholamines were determined from the radioactivity of the 3-methyl derivatives, since 3-O-methylation proceeds preferentially under the reaction conditions used. The radioactivity was corrected by the recoveries of methyl derivatives added as carriers. Standard curves of norepinephrine, epinephrine and dopamine were obtained between 4 pg and 48 pg per 50 μl (Fig. 5). Rates of formation of 3-methyl derivatives were calculated from specific activity of

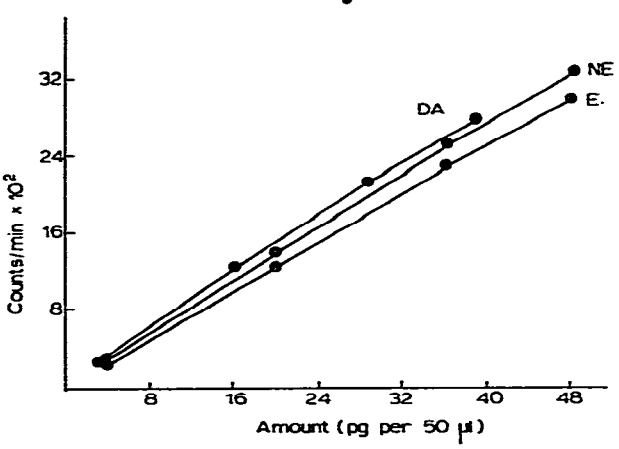

Fig. 5. Standard curves of catecholamines. NE = Norepinephrine; E = epinephrine; DA = dopamine.

TABLE II

RECOVERY OF NE, E AND DA ADDED TO DEPROTEINIZED SERUM

Amount added (pg per 50 µl)	Found (pg per 50 µl)*		
	NE	E	DA
8	8	8	7
16	15	14	16
24	25	26	24
40	38	41	40

*Endogenous catecholamines are subtracted.

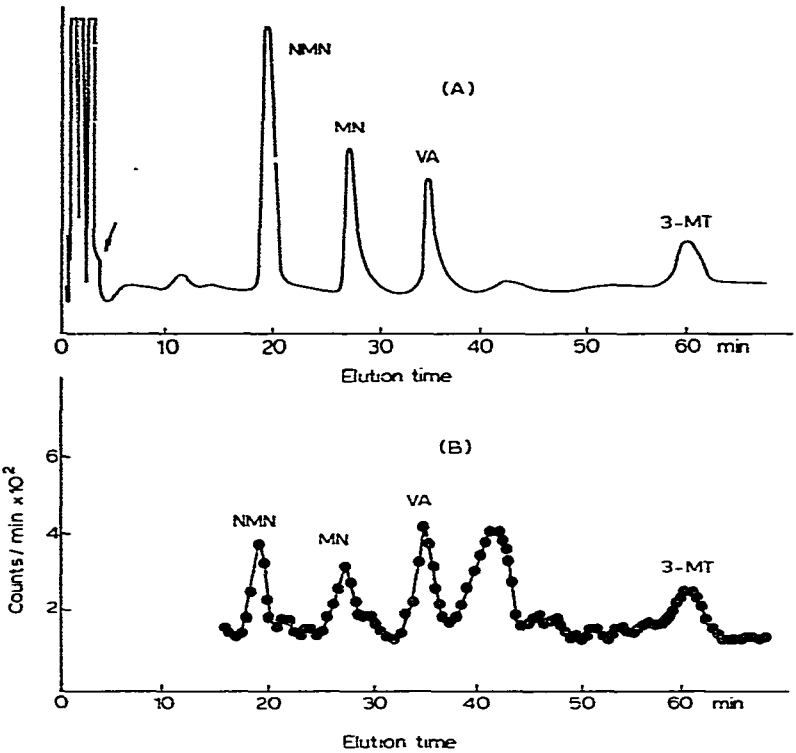

Fig. 6. Chromatograms of O-methylated serum catecholamines (A, absorbance at 254 nm; B, ^{3}H radioactivity). Sample was a quarter of the methylated products from 50 μl of deproteinized serum. Other conditions as in Fig. 4.

TABLE III

DETERMINATION OF SERUM CATECHOLAMINES

Serum sample	Amount (pg/ml)		
	NE	E	DA
A	516	177	244
B	236	147	144
C	436	154	177
	404	185	170
	423	170	165

[^{3}H]SAM, and were 60, 60 and 57% for norepinephrine, epinephrine and dopamine, respectively, at a concentration of 20 pg per 50 μl. The rates decreased slightly with increase in catecholamine concentration.

The procedure was applied to the analysis of serum catecholamines (Fig. 6). Although the radioactivity of the baseline fractions increased more than two times, distinct peaks corresponding to three catecholamine derivatives were observed. The peak eluting after VA has approximately the same retention time as MTA and 4-ME; however, it also contains an unidentified component from serum. The recoveries of catecholamines added to 50 μl of deproteinized

serum were practically quantitative between 8 and 40 pg (Table II). This indicated that standard curves for artificial mixtures were applicable to the analysis of serum. Results of analysis of sera from three healthy adults are listed in Table III. Serum C was analysed three times and the relative average deviations were between 3 and 6%. The values in Table III were in the range of catecholamine concentrations reported for healthy adults [7, 16, 17].

DISCUSSION

Since this procedure was designed for routine analysis of catecholamines, chemical reactions like periodate oxidation [6] and cumbersome manipulations like solvent extraction and thin-layer chromatographic separation [12] were avoided. Instead, we used boric acid gel column and liquid chromatography for isolation of the methyl derivatives.

Catecholamines are known to be methylated by COMT at both the 3 and the 4 positions on the catechol ring and the relative quantity of the two isomers depends on the reaction conditions and the nature of the enzyme [18, 19]. However, little consideration has been given to this in radioenzymatic assay procedures previously reported. In the present procedure, substantial amounts of 4-methyl derivatives were produced. The relative quantity of 4-methyl derivatives was not significantly reduced by using rat liver COMT in a commercially available catecholamine assay kit, KAT-A-KIT. The rate of formation of 4-methyl derivatives in the enzyme reaction was reported to be independent of catecholamine concentration [20], but it seems to decrease at extremely low concentrations, although they were always detectable. From these observations it can be seen that the isomers should be separated from the methyl derivatives for accurate determination of each catecholamine, as in the present procedure.

The sensitivity of radioenzymatic assay depends on several factors. These are the specific activities of isotope derivatives, rate of enzyme reaction, recovery of products during isolation and signal-to-noise ratio in the determination. In the present procedure, we used [^{3}H] SAM, tritiated about 90%, for the determination of picogram levels of catecholamines; we obtained about 60% of catecholamines as 3-methyl derivatives and reduced the contaminating radioactivity to ca. 120 cpm including natural background. Consequently, it is still possible to increase the sensitivity by finding the reaction conditions under which 3-methyl derivatives are produced quantitatively and by removing all the contaminating radioactivity from reaction products.

In conclusion, an accurate and sensitive procedure for the determination of catecholamines has been developed. The procedure requires little expertise by the use of boric acid gel column and ion-pair liquid chromatography with a branched flow path.

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