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EFFECTS OF L-DOPA ON DOPAMINE AND NOREPINEPHRINE CONCENTRATIONS IN RAT BRAIN ASSESSED BY GAS CHROMATOGRAPHY

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SUMMARY

A highly specific and sensitive gas chromatographic method has been developed which is capable of determining picogram amounts of dopamine (DA) and norepinephrine (NE) simultaneously. The catecholamines are converted to the N-2,6-dinitro-4-trifluoromethylphenyl, O-trimethylsilyl derivatives, which are analyzed by gas chromatography with electron-capture detection. The method has been applied to the assay of catecholamines in rat brain extracts. One hour after an acute dose (150 mg/kg i.p.) of L-3,4-dihydroxyphenylalanine, the rat brain concentration of DA increased by 130% while the concentration of NE was unchanged.

INTRODUCTION

The therapeutic effects of L-3,4-dihydroxyphenylalanine (L-DOPA) in Parkinson's disease are generally assumed to be due to the conversion of the catechol amino acid into dopamine (DA) in the basal ganglia[1]. The extent to which the DA is converted further to norepinephrine (NE) and the relative importance of NE formation to the effects of L-DOPA is not clear.

While studies on L-DOPA administration in experimental animals have shown a marked elevation in brain DA concentration[2], contradictory results have been reported for the effects on NE metabolism. Some investigators have shown that L-DOPA treatment does not elevate NE concentration in whole brain of rats[3,4] or mice[5] and may even decrease rat brain NE levels[6]. In contrast, other studies have reported that L-DOPA results in an increase in the NE content of the rat brain[7] and the cat brain stem[8] and spinal cord[9]. Although the cause of this apparent discrepancy is not clear, it may be accounted for, in part, by differences in the dosage and the method of administration of L-DOPA, species or brain regions. On the other hand, since these studies were carried out using fluorometric techniques for the analysis of NE, the possibility exists that high levels of L-DOPA or its metabolites might interfere with the measurement of NE.

In the present paper, we describe a highly sensitive and specific gas chromatographic (GC) method based on the use of 2,6-dinitro-4-trifluoromethylbenzenesulfonic

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acid (DNTS) as a derivatizing reagent[10] for the simultaneous measurement of DA and NE in tissues. Using this method, we have reinvestigated the influence of L-DOPA on catecholamines in rat brain.

EXPERIMENTAL

Animals and materials

Seven-week old male Sprague-Dawley rats (Zivic-Miller Laboratories, Pittsburgh, PA, U.S.A.) weighing 245–295 g were used. The hydrochloride salts of dopamine and norepinephrine were obtained from Sigma (St. Louis, MO, U.S.A.). The hydrobromide salt of α-methyldopamine (MDA) was kindly provided by Sterling Winthrop Research Institute (Rensselaer, NY, U.S.A.). The hydrochloride salt of α-methylnorepinephrine was purchased from Regis (Chicago, IL, U.S.A.). L-3,4-Dihydroxyphenylalanine and the hydrobromide salt of 3,4-dihydroxybenzylamine were purchased from Sigma. N,O-Bis(trimethylsilyl)acetamide was obtained from Pierce (Rockford, IL, U.S.A.). 4-Chloro-3,5-dinitrobenzotrifluoride was purchased from K & K Labs. (Plainview, NY, U.S.A.) and Tris(hydroxymethyl)aminomethane (Ultrol grade) from Calbiochem (Somerville, NJ, U.S.A.). Aluminum oxide (Woelm Neutral Activity Grade I) was obtained from ICN Pharmaceuticals (Cleveland, OH, U.S.A.). Pesticide grade ethyl acetate and benzene were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). Sodium 2,6-dinitro-4-trifluoromethylbenzenesulfonate was synthesized by the reaction between 4-chloro-3,5-dinitrobenzotrifluoride and sodium sulfite following the method of Gerig and Reinheimer[11].

Extraction of catecholamines from brain tissues

Two groups of rats received intraperitoneal (i.p.) injections of either L-DOPA (150 mg/kg) (dissolved in 0.05 *N* HCl by mild heating) or the diluent (1 ml/100 g). The animals were sacrificed by decapitation 1 h after injection. The brains were quickly removed, blotted and frozen on dry ice. The next day, the brain tissues were homogenized for 20 sec in 10 volumes of cold 0.4 *N* perchloric acid using a Polytron homogenizer. The homogenates were centrifuged at 27,000 *g* for 10 min at 4°C. A 10-ml aliquot of each supernatant was transferred to a 15-ml screw-cap tube. Supernatants obtained from 6 brain homogenates of untreated rats were pooled and to separate 10-ml aliquots, various amounts (0–1400 ng) of DA and NE were added for standards. Then 16 μg of the internal standard, MDA, were added to each sample and standard. The tubes were inverted five times for mixing, and 0.5 ml of 10% EDTA disodium salt and 0.1 ml of freshly prepared 5% sodium metabisulfite were added with additional mixing. By means of a pre-calibrated scoop, 640 mg of alumina (washed and neutralized according to the procedure of Anton and Sayre[12]) was added to each tube followed by 1 ml of cold 1 *M* Tris buffer, pH 8.4. After mixing by tube inversion, the pH of each solution was adjusted to pH 8.4 with 5 and 1 *N* NaOH. The tubes were shaken by inversion for 10 min and centrifuged at 750 *g*. The supernatant was discarded by aspiration and the alumina washed twice by adding 10 ml of cold water followed by inverting 30 times and discarding the supernatant. The adsorbed catecholamines were then eluted by mixing the alumina for 5 min with 3 ml of cold 0.2 *N* acetic acid. The eluate was transferred to a 15-ml conical-bottom tube and lyophilized.

Derivative formation

The catecholamines were then converted to the N-2,6-dinitro-4-trifluoromethylphenyl (N-DNT) derivatives by adding 0.5 ml of 0.11 *M* DNTS in 10% sodium borate, pH 9.3. After the samples had reacted for 10 min at room temperature, the N-DNT catecholamines were extracted in two 0.4-ml portions of ethyl acetate. Following centrifugation at 750 *g* for 5 min, the organic phases were transferred to acid-washed, silanized 0.3-ml Reacti-Vials (Pierce) and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 100 μl of ethyl acetate and flushed with nitrogen. The hydroxyl groups of the N-DNT catecholamines were then trimethylsilylated by adding 10 μl of N,O-bis(trimethylsilyl)acetamide and heating at 60°C for 15 min. After cooling the reaction mixture to room temperature, the solvent and excess reagent were removed by evaporation under a stream of nitrogen. The N-DNT, O-trimethylsilyl (O-TMS) derivatives were then dissolved in 0.4 ml of benzene since salts and other components that were removed by the ethyl acetate extraction were found to interfere with the GC analysis. The benzene solution was diluted 40-fold and 0.5–1.0 μl was injected into the gas chromatograph.

Gas chromatography

A Packard Model 417 gas chromatograph equipped with a ^{63}Ni electron-capture detector was used. A 6 ft. × 2 mm I.D. silanized glass column packed with 3% OV-1 on 80–100 mesh Supelcoport (Supelco, Bellefonte, PA, U.S.A.) was used with ultrapure nitrogen as the carrier gas at a flow-rate of 30 ml/min. The column temperature was maintained at 240°C and the detector and inlet port at 260°C.

Gas chromatography–mass spectrometry

The chemical-ionization mass spectra of N-DNT, O-TMS catecholamines were obtained on a Finnigan Model 3200 combined gas chromatograph–mass spectrometer. The gas chromatograph was equipped with a 5 ft. × 2 mm I.D. silanized U-shaped glass column packed with 3% OV-1 on 80–100 mesh Supelcoport. The operating conditions were: column temperature, 250°C; injection port temperature, 270°C; electron multiplier voltage, 1900 V; electron energy, 120 eV; emission current, 0.50 mA; ion source pressure, 1.0 Torr; reagent gas (methane) flow-rate, 10 ml/min.

Quantitation

The amount of DA and NE in each unknown sample was quantitated from the ratio of peak heights of the catecholamine and the internal standard, MDA, and comparing this ratio with the calibration curves obtained for standards carried through the extraction scheme.

RESULTS

The retention times of the N-DNT, O-TMS derivatives of some catecholamines are given in Table I. Since MDA and 3,4-dihydroxybenzylamine were completely resolved from DA and NE, either could be utilized as an internal standard. However, under the GC conditions employed, α-methylnorepinephrine was not resolved from DA. The derivatives of 3,4-dihydroxybenzylamine, α-methylnorepinephrine and MDA were about 3 to 4 times less sensitive towards electron-capture detection than the derivative of DA.

TABLE I

CHROMATOGRAPHIC RETENTION DATA OF N-DNT, O-TMS CATECHOLAMINES

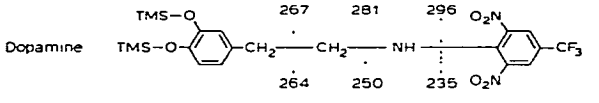
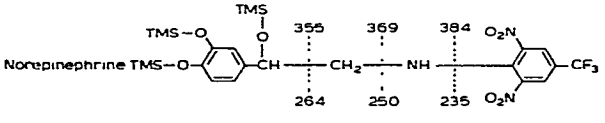
GC conditions were the same as mentioned in the Experimental section except that the carrier gas flow-rate was 27 ml/min.

Catecholamine	*Retention time (min)*	*Relative retention time*
α-Methyldopamine	2.95	1.00
3,4-Dihydroxybenzylamine	3.10	1.05
α-Methylnorepinephrine	3.78	1.28
Dopamine	3.89	1.32
Norepinephrine	4.49	1.52

The proposed structures of the catecholamine derivatives were confirmed by their chemical-ionization (methane) mass spectra (Table II). The base peaks for the derivatives of DA and NE appeared at *m/e* 281 and *m/e* 355, respectively.

TABLE II

CHEMICAL-IONIZATION (METHANE) MASS SPECTRA OF N-DNT, O-TMS CATECHOLAMINES

Catecholamine	*Structure of derivative and probable fragmentation pattern*	*m/e values of major fragment ions and their relative abundances (%)*
Dopamine		512 (20%, MH^+ − HF) 298 (14%, MH^+ − 235 + H) 281 (100%, MH^+ − 250 − H) 267 (26%, MH^+ − 264 − H) 264 (6%, MH^+ − 267 − H) 252 (4%, MH^+ − 281 − H) 246 (2%, MH^+ − 267 − F) 232 (10%, MH^+ − 281 − F) 219 (4%, MH^+ − 267 − NO_2) 210 (5%, MH^+ − 235 − TMSOH + 3H) 201 (4%, MH^+ − 267 − F − NO_2 + H)
Norepinephrine		600 (28%, MH^+ − HF) 530 (33%, MH^+ − TMSOH) 510 (22%, MH^+ − TMSOH − HF) 369 (17%, MH^+ − 250 − H) 355 (100%, MH^+ − 264 − H) 298 (56%, MH^+ − 235 − TMSOH + 3H) 281 (72%, MH^+ − 250 − TMSOH + H) 267 (30%, MH^+ − 264 − TMSOH + H) 264 (9%, MH^+ − 355 − H) 246 (39%, MH^+ − 355 − F) 232 (30%, MH^+ − 369 − F) 219 (11%, MH^+ − 355 − NO_2) 210 (15%, MH^+ − 235 − 2TMSOH + 5H) 201 (15%, MH^+ − 355 − F − NO_2 + H)

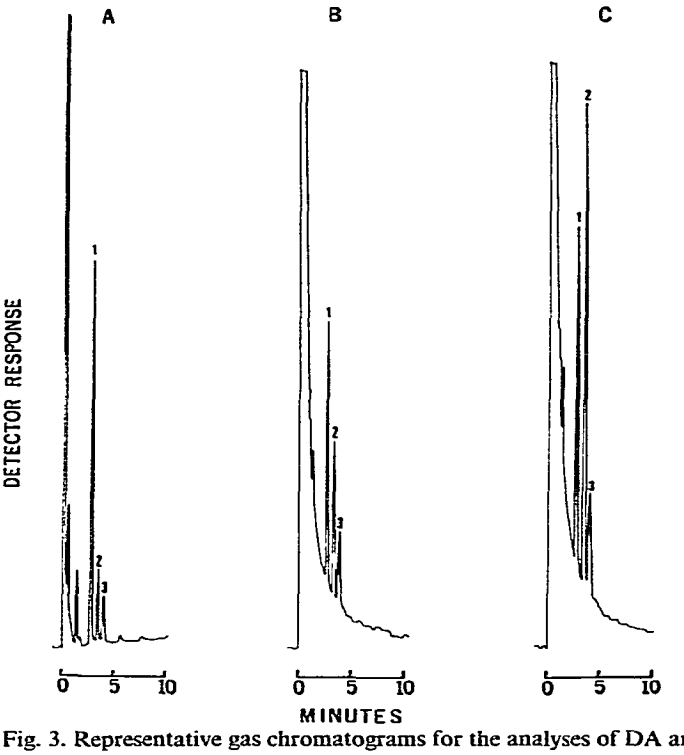


Fig. 3. Representative gas chromatograms for the analyses of DA and NE. A, Unextracted standards; B, brain from a control rat; C, brain from a rat treated with 150 mg/kg of L-DOPA 1 h before sacrifice. Peaks: 1 = internal standard, MDA; 2 = DA; 3 = NE. The standard mixture represents 103 pg of MDA and 6 pg each of DA and NE.

The calibration curves for DA and NE, using MDA as an internal standard, are shown in Figs. 1 and 2, respectively.

Fig. 3 illustrates the GC separation of DA and NE in whole brains from a control and an L-DOPA treated rat.

The rat brain catecholamine concentrations in control and L-DOPA treated rats are shown in Table III. The mean steady state levels of DA and NE in brains from control rats were 755 ± 28 ng/g and 286 ± 12 ng/g, respectively. In the group of rats receiving L-DOPA (150 mg/kg i.p.) 1 h before sacrifice, the mean concentration of brain DA was 1737 ± 65 ng/g (230% of the control group), while the brain concentration of NE was unchanged (294 ± 19 ng/g).

TABLE III

RAT BRAIN CATECHOLAMINE CONCENTRATIONS ONE HOUR AFTER A SINGLE INJECTION OF 150 mg/kg OF L-DOPA

Values are the mean ± S.E.M.

Group	*Dopamine (ng/g)*	*Norepinephrine (ng/g)*
Control (n = 6)	755 ± 28	286 ± 12
L-DOPA (n = 5)	1737 ± 65*	294 ± 19

* $p < 0.001$ compared to control group using a two-tailed Student's t-test.

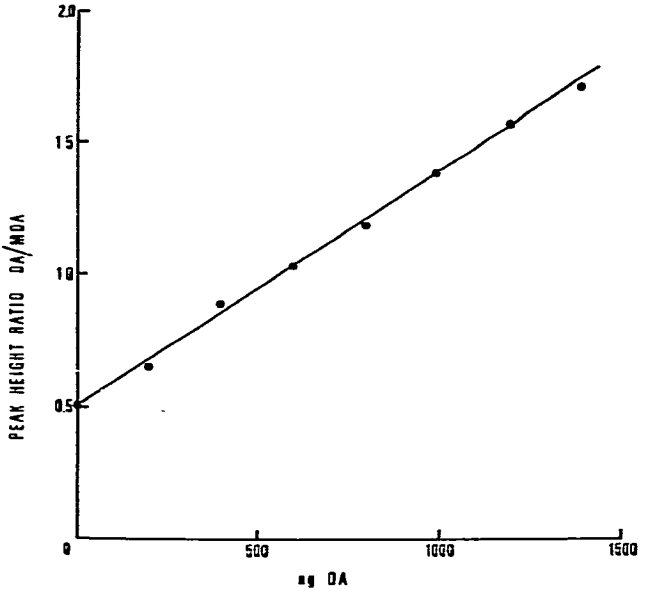


Fig. 1. Plot of peak height ratio, DA/MDA, *vs.* amount of DA added to aliquots of a pooled supernatant solution obtained from brains of untreated rats. Correction was made by subtracting the *y*-intercept which represents the amount of endogenous DA present in the supernatant.

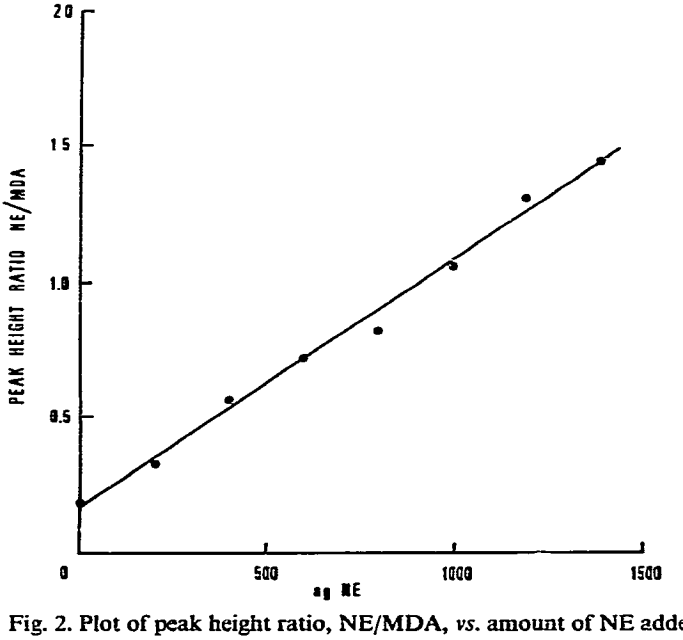


Fig. 2. Plot of peak height ratio, NE/MDA, *vs.* amount of NE added to aliquots of a pooled supernatant solution obtained from brains of untreated rats. Correction was made by subtracting the *y*-intercept which represents the amount of endogenous NE present in the supernatant.

DISCUSSION

We have developed a GC method with electron-capture detection for the simultaneous analysis of DA and NE in brain tissue. The sensitivity of the method appears to be at least as good as the other existing assays for catecholamines. For example, the minimal amount of DA or NE injected on column that could be detected was 0.5 pg (signal-to-noise ratio of 3). For comparison, the minimal detectable levels of catecholamines analyzed by high-performance liquid chromatography with electrochemical detection is in the range of 2–100 pg[13,14].

We have applied our GC method for studying the effects of L-DOPA on rat brain catecholamine levels. We found that an acute injection of L-DOPA (150 mg/kg) caused a 130% increase in rat brain DA concentrations but no effect on NE concentrations. While this is in agreement with a few studies[4,5], it is contrary to other studies that have reported increases in brain NE levels following an injection of L-DOPA[7,8]. Although we cannot be certain of the cause of these discrepancies, the fact that virtually all previous studies utilized fluorometric methods suggests the likelihood that the lack of total specificity of the fluorometric assays may be responsible for the discrepancies in results.

Although our studies indicate that L-DOPA does not increase the steady state NE concentration in brain, this does not imply that L-DOPA has no effect on NE metabolism. For example, recent studies in our laboratory have revealed that levels of 3-methoxy-4-hydroxyphenylethyleneglycol (MHPG), a major metabolite of NE in brain, are increased by about 70% 1 h following an i.p. injection of 150 mg/kg of L-DOPA, the same dose that was used in the present study[15]. Using deuterium-labelled L-DOPA, it was demonstrated that essentially all the increase in MHPG levels was accounted for by the conversion of L-DOPA to NE. Taken together with the present results, we conclude, therefore, that the effects of L-DOPA on NE metabolism in brain cannot be assessed solely by the steady state NE levels.

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