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QUANTITATIVE THIN-LAYER CHROMATOGRAPHIC ESTIMATION OF LABELED DOPAMINE AND NOREPINEPHRINE, THEIR PRECURSORS AND METABOLITES*

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SUMMARY

A simple quantitative separation method for combinations of sixteen ^{14}C- and ^{3}H-labeled catecholamines, their precursors and metabolites has been devised. The method uses precoated cellulose plates, diazotized *p*-nitroaniline as a spray detection agent and quantitates the labeled compounds by means of a liquid scintillation counter. The efficiency of the method was tested by examining differences of *in vitro* and *in vivo* metabolism of ^{14}C-labeled dopamine by control and enzyme-inhibited caudate nucleus.

INTRODUCTION

Thin-layer chromatographic (TLC) methods have been described for the separation and detection of catechol biogenic amines which permit rapid isolation and identification of small amounts of these compounds. However, the methods available are limited in the number of amines, their metabolites and precursors that can be examined and some are not suitable for use with radioisotopes[1-6].

This report, an extension of our previous publication[7], concerns a new two-dimensional TLC separation of a combination of the following compounds: DA, DHMA, DHPG, DOPA, DOPAC, EPI, HVA, MET, MHPG, 3MT, NE, NMN, OCT, TYM, TYR, and VMA**. The system uses precoated cellulose plates and allows a simultaneous, quantitative estimation of unconjugated ^{14}C- and ^{3}H-labeled compounds by means of a liquid scintillation counter. The method has been successfully applied to the analysis of DA metabolism in tissues such as: biopsies or tissue cultures of caudate nucleus and substantia nigra from rats, brain biopsies from patients

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** DA = dopamine, DHMA = 3,4-dihydroxymandelic acid; DHPG = 3,4-dihydroxy-phenylglycol; DOPA = 3,4-dihydroxyphenylalanine; DOPAC = dopacetic acid; EPI = L-epinephrine; HVA = homovanillic acid; MET = metanephrine; MHPG = 3-methoxy-4-hydroxyphenylglycol; 3MT = 3-methoxytyramine; NE = L-norepinephrine; NMN = normetanephrine; OCT = octopamine; TYM = tyramine; TYR = tyrosine; VMA = 3-methoxy-4-hydroxymandelic acid.

with Parkinson's disease and drug-induced dyskinesia models in rats and monkeys, all of which were incubated with labeled substrates. Thus it provides a simple radiometric assay for several enzymes involved in amine metabolism.

MATERIALS AND METHODS

Reference standards

The unlabeled reference standards were all from commercial sources and dissolved in 0.01 *N* HCL to make concentrations of 1 mg/ml. The μg amounts usually spotted on the chromatoplates were: DA (1), DHMA (1), DHPG (2.5), DOPA (0.5), DOPAC (1), EPI (1), HVA (1), MET (0.5), MHPG (1), 3MT (1), NE (1), NMN (0.5), OCT (0.5), TYM (1), TYR (1), and VMA (0.5). The ^{14}C-labeled DA (38 mCi/mmole) and ^{3}H-labeled DA (280 mCi/mmole) were purchased from Nuclear Chicago and dissolved in 0.01 *N* HCl.

Precoated plates

The 20 × 20 cm plates used were Avicel microcrystalline cellulose powder, glass backing, without fluorescent indicator (Merck, Darmstadt, distributed by Brinkmann Instrument Co., Westbury, N.Y., U.S.A.), 80 μ thick, or Cellulose Chromagram Sheet No. 6064, plastic backing, without fluorescent indicator (Eastman Co., Rochester, N.Y.), 160 μ thick. The Brinkmann plates were superior in quality and gave more uniform separation.

Solvents and solutions

All solvents and solutions used were analytical grade; 1-butanol, absolute methanol, and chloroform were obtained from Baker, ammonium hydroxide, formic acid, and glacial acetic acid from Mallinckrodt.

Detection reagent

DPNA* prepared from (a) *p*-nitroaniline (Eastman) 0.1 g dissolved in 2 ml concentrated HCl and diluted to 100 ml with water, (b) $NaNO_2$ (Mallinckrodt) 0.2 g dissolved in 100 ml water, and (c) K_2CO_3 (Mallinckrodt) 10 % solution, 20 g dissolved in 200 ml water. Equal volumes (usually 1 ml each) *p*-nitroaniline and $NaNO_2$ were placed in a beaker packed in ice. After 10 min 2 vol. K_2CO_3 solution were added to the beaker and mixed. The spray was applied to the plate using a small atomizer at a distance of 10 cm until the plates appeared translucent. Excess of reagent was avoided to preclude any diffusion of the spot and radioactivity.

Counting fluorophore

The scintillation counting fluorophore consisted of (a) naphthalene (100 g) scintillation grade, (b) PPO* (5 g), (c) POPOP* (0.2 mg) (all Packard Instrument Co.), and (d) *p*-dioxane (1 l) spectroquality (Mathison, Coleman and Bell). In a refrigerated counter the fluorophore had a tendency to freeze after several hours at the reduced temperature of the freezer.

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* DPNA = diazotized *p*-nitroaniline; PPO = 2,5-diphenyloxazole; POPOP = 1,4-bis-(5-phenyloxazolyl-2)-benzene.

Developing tanks

Glass, 10.75 × 9.25 × 2.75 in. inside dimensions, with lid (Kensco). Solutions of the sixteen reference standards were applied with a capillary to the cellulose chromatoplate at a point 1.5 cm from the base. The plates were developed in 2 dimensions with the solvent systems 1-butanol–methanol–1 *N* formic acid (60:20:20) in direction 1 and chloroform–methanol–1 *N* ammonium hydroxide (60:35:5) in direction 2. Between directions and after development, the plate was dried with a hair drier using warm air. The time for ascending development varied with the solvent system used, 3.5–4 h in direction 1 (plate layered direction) and 1.5–2 h in direction 2, for a height of 13 cm from the origin. The reference standards developed characteristic colors after exposure to DPNA spray. Lining the tank walls with filter paper moistened with solvent or saturating the chamber atmosphere did not enhance the separation.

When labeled compounds were used in this procedure the basic separation conditions were the same. However, radioactive standards or tissue extracts containing labeled material were applied to the origin along with the cold standards. After the separation and identification of spots with DPNA, the standard colored areas were scraped off and placed in individual counting vials containing 0.5 ml water. The scraped powder was gently swirled in the water and then 10 ml counting fluorophore was added and the samples were counted in a Packard liquid scintillation counter. The elution of the radioactivity from the cellulose into the fluorophore was 100 %.

RESULTS AND DISCUSSION

Separation and recovery

The R_F values of each compound and the spray-developed colors are presented in Table I. The values were obtained at 22° and represent the center of the spot. The separation of DA, its precursors and metabolites, is shown in Fig. 1. The spots remained small and round and there was no trailing of any of the standards. The amino acid DOPA has the lowest R_F value, the amines intermediate values and the acid metabolites the highest R_F values. A similar separation was observed for NE and its metabolites, with the acid and glycol metabolites having the highest R_F values (Fig. 2). Eleven compounds of interest in Parkinson's disease, drug-induced dyskinesias, and DOPA therapy can be separated well using this method (Fig. 3). However, it is difficult to separate DA from EPI and 3 MT from MET if these NE metabolites are present in a DA sample.

The method was examined for quenching effect and extraction recovery after separation. Because the labeled derivatives of many of the compounds in this study were not available, quenching was tested by adding 1000 c.p.m. each of ^{14}C-DA and ^{3}H-DA to the sixteen unlabeled reference standards that had been spotted on TLC in 32 separate 1 × 1 cm squares in amounts of 2–10 μg. After spraying and scraping, the individual samples were counted. The presence of the DPNA-sprayed standards did not appreciably affect the ^{14}C-DA since there was less than an average 5 % quenching of the sixteen compounds. However, the ^{3}H-DA was quenched an average of 63 %. Thu sthe present spraying and counting technique has limitations when using ^{3}H. Exposure of the developed plate to paraformaldehyde

TABLE I

R_F VALUES AND COLOR DEVELOPMENT OF REFERENCE STANDARDS

Solvents: direction 1, 1-butanol–methanol–1 *N* formic acid (60:20:20); direction 2, chloroform–methanol–1 *N* ammonium hydroxide (60:35:5).

Reference standard	*R_F value*		*Color after spraying with DPNA*
	Direction 1 (4 h)	*Direction 2 (2 h)*	
DA, its precursors and metabolites (see Fig. 1)			
DOPA	0.22	0.02	Magenta
TYR	0.38	0.08	Pink
DA	0.45	0.24	Magenta
OCT	0.50	0.31	Red
3MT	0.55	0.58	Blue
TYM	0.60	0.52	Pink
DOPAC	0.82	0.07	Magenta
HVA	0.90	0.34	Blue
NE, its precursors and metabolites (see Fig. 2)			
NE	0.35	0.12	Magenta
EPI	0.40	0.25	Magenta
NMN	0.45	0.41	Purple
MET	0.49	0.60	Purple
DHMA	0.60	0.05	Magenta
VMA	0.72	0.17	Purple
DHPG	0.68	0.56	Magenta
MHPG	0.68	0.71	Purple

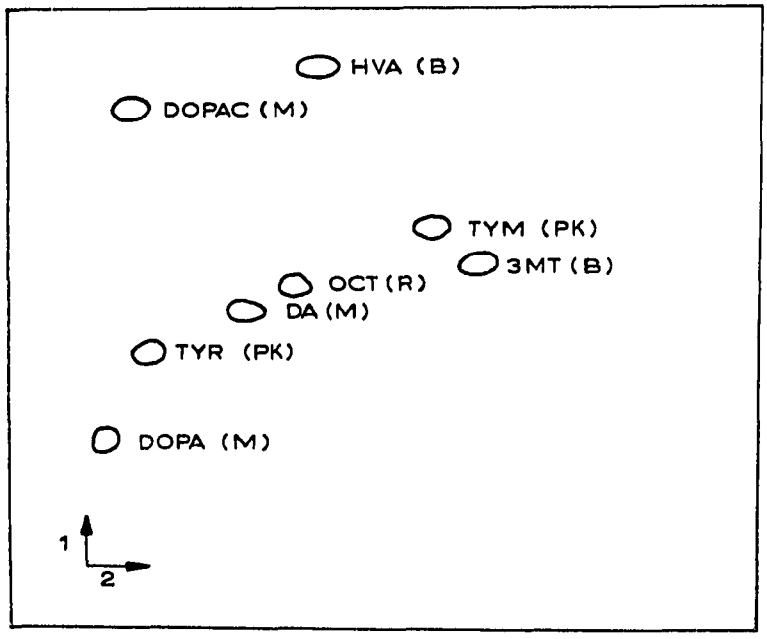

Fig. 1. Separation of DA, its precursors and metabolites. Plate: Eastman Cellulose No. 6064, without fluorescent indicator (160 μ), or Brinkmann microcrystalline cellulose (80 μ). Time: direction 1, 3.5–4 H; direction 2, 1.5–2, H. Solvents: direction 1, 1-butanol–methanol–1 *N* formic acid (60:20:20); direction 2, chloroform–methanol–1 N ammonium hydroxide (60:35:5). Spray: DPNA. Color development: M = Magenta, PK = pink, R = red, B = blue, and P = purple

gas and UV to visualize the standards by fluorescence[7] and/or autoradiography to locate the ^{3}H-labeled material can be used to eliminate the limitations caused by the quenching effect.

The elution recovery of pure ^{14}C-DA, ^{14}C-DOPA, and ^{14}C-NE standards separated and developed on TLC was uniformly 90% or better. Autoradiography was used to check the efficacy of scraping only the spray-developed reference

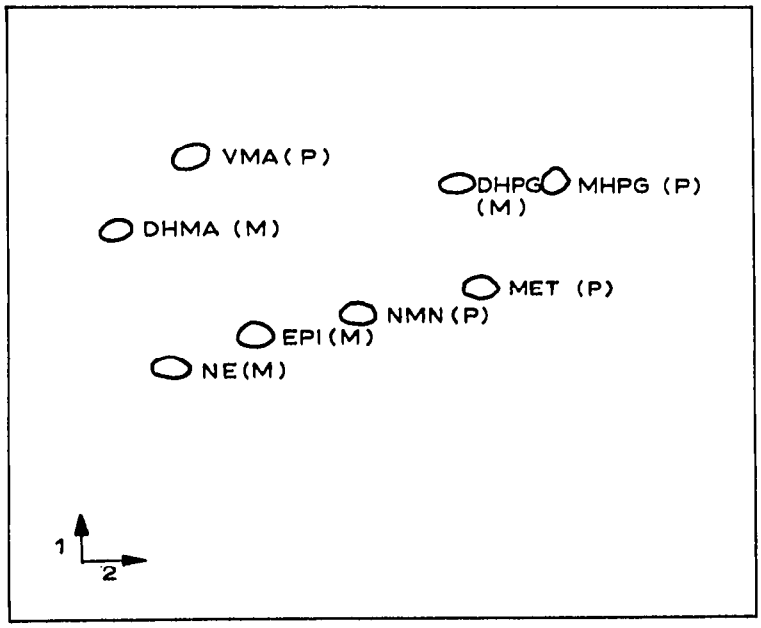

Fig. 2. Separation of NE and its metabolites. For conditions see legend to Fig. 1.

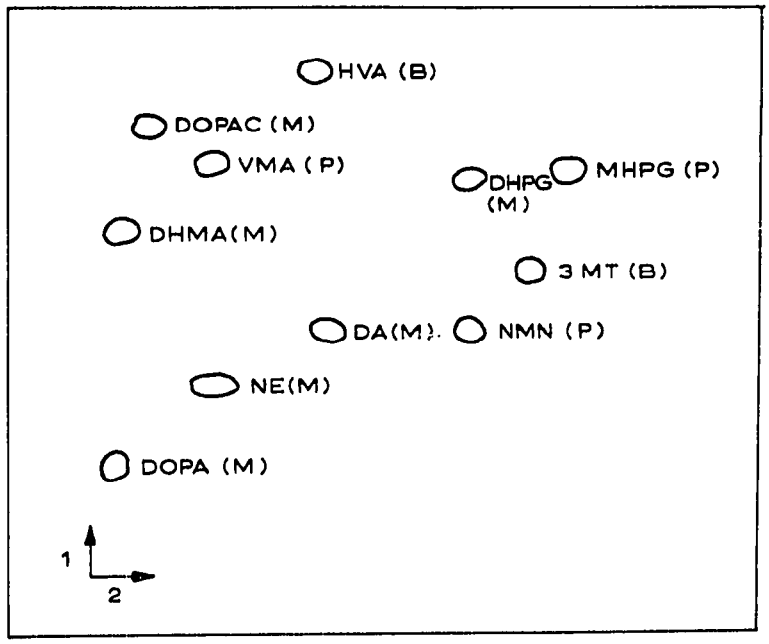

Fig. 3. Separation of compounds of interest in Parkinson's disease, drug-induced dyskinesias, DOPA therapy, etc. For conditions see legend to Fig. 1.

standard area. 91 % of ^{14}C counts applied from an *in vivo* mouse caudate nucleus experiment were recovered in the immediate area of the developed reference standards. The autoradiography showed some radioactivity on the periphery of the spots, so for complete recovery it was advisable in all experiments to scrape a short distance (1 mm) beyond the standard spot area.

Biological application

An application of the TLC separation to the metabolism of ^{14}C-DA by caudate nucleus showed metabolic differences in the rat *in vivo* and *in vitro*. Also, by inhibition of monoamine oxidase (MAO) and alteration of the normal caudate nucleus metabolism of DA *in vivo*, it was possible to show differences in metabolic products and give validity to the premise that the labeled substances formed were the metabolites mentioned.

For the *in vitro* studies, caudate nuclei were quickly dissected at 4° from brains of Sprague–Dawley rats sacrificed by decapitation. Slices not more than 0.5 mm thick were made from the caudates and an equal number of slices (approximately 50 mg) were placed in 5-ml vessels which contained 1 ml of Krebs phosphate medium[8], pH 7.4 gassed with 5 % CO_2–95 % O_2. After 15-min preincubation for temperature equilibration, the tissues were incubated for 20 min with 5×10^5 c.p.m. ^{14}C-DA. The tissues were removed from the vessels, rinsed in cold saline and homogenized with a Bronwill Biosonic Sonifier (Bronwill Scientific, Rochester, N.Y., U.S.A.) in 0.2 ml 0.2 *N* acetic acid which contained 1 μg of each reference standard of interest in the DA study. Aliquots were taken of the total homogenate and the centrifuged supernatant for counting, in order to measure ^{14}C-DA uptake and tissue extraction recovery. Aliquots of 10–25 λ of the tissue supernatant and Krebs medium, which had two drops of glacial acetic acid and 1 μg of each reference standard added, were spotted on the cellulose chromatoplate along with 1 μg DA and metabolite reference standards. The plates were developed and the radioactivity counted as described in MATERIALS AND METHODS. The lateral ventircle freehand injection technique of CLARK *et al.*[9], developed for mice, was adapted for use with rats under light ether anesthesia in the MAO inhibition study. In the examination of *in vivo* caudate nucleus metabolism of DA, 15 min after the injection of 2.5×10^5 c.p.m. ^{14}C-DA, in 5 λ pH 7.0 Krebs solution, into each ventricle, the rats were sacrificed by decapitation and the caudate nucleus rapidly removed at 4°. The tissue was rinsed in cold saline, weighed on a microbalance and frozen immediately over dry ice until analyzed, which was within 15 h of sacrificing.

The frozen tissue was broken up by the sonifier in 0.2 ml 0.2 *N* acetic acid after the addition of 1 μg DA and metabolite reference standard. Aliquots of this homogenate and the resulting supernatant, after centrifugation at $10{,}000 \times g$ and 0°, were taken for ^{14}C-DA uptake and tissue extraction data. Aliquots were spotted on the cellulose TLC as in the *in vitro* experiments mentioned above.

The results are shown in Table II. The relative amount of each compound is expressed as a percentage of the radioactivity of all the reference standard areas counted. Because of the small *N* value in this experiment it is not possible to include statistical differences between *in vivo* and *in vitro* metabolism of ^{14}C-DA by rat caudate nucleus but certain observations can be made.

The major metabolite found *in vitro* in both the tissue and in the Krebs medium

TABLE II

In vitro AND *in vivo* METABOLISM OF ^{14}C-DA BY RAT CAUDATE NUCLEUS

Reference standards	*Average % of recovered ^{14}C found in reference standards of interest, combined to total 100%*		
	In vitro[a]		*In vivo*[b] ($N = 2$)
	Tissue ($N = 3$)	*Medium* ($N = 3$)	
Origin	5.2 Range (1.7–9.3)	1.0 (0.9–1.2)	1.2 (0 2.5)
NE	0.4 (0–0.9)	0.9 (0.5–1.3)	1.9 (1.0–2.8)
DA	82.7 (74.5–93.2)	81.7 (74.5–89.1)	57.3 (52.1–63.6)
3 MT	0.2 (0–0.5)	0.2 (0–0.5)	5.1 (4.3–5.9)
DOPAC	11.6 (5.0–15.4)	14.8 (8.6–20.8)	13.4 (13.2–13.7)
HVA	0.1 (0–0.3)	1.4 (0.3–2.5)	22.5 (20.5–24.5)

[a] Incubated 20 min: 10–25 λ spotted on TLC plate.
[b] Sacrificed by decapitation 15 min after lateral ventricle injection.

was DOPAC. This has been reported by others in bovine[10] and rat brain homogenates[4,10] and rabbit caudate nucleus[11]. There was little HVA, 3 MT or NE formed. The apparent absence of labeled NE is interesting since UDENFRIEND AND CREVELING[12] found the caudate nucleus of two species (dog and bovine) to be almost as high in dopamine-β-oxidase activity as hypothalamus and comparable with the adrenal medulla. However, others have reported negligible conversion of labeled precursors to ^{14}C-NE by caudate nucleus of cat[13], rabbit[11] and rat brain homogenate[4].

The *in vivo* metabolic pattern is somewhat different. HVA was found to be the major catabolite, followed in decreasing order by DOPAC, 3 MT, and NE. In support of our findings, LAVERTY AND TAYLOR[14], using intraventricular administration of ^{3}H-DA, reported a metabolic pattern for the rat striatum, 1 h post injection which is similar to ours, with HVA the major catabolite. Also, in a review dealing with DA and brain function, HORNYKIEWICZ[15] stated that HVA is the main metabolic breakdown product of DA in brain.

To test the efficiency of this method further, we used a rapidly acting MAO inhibitor which has a strong effect on DA metabolism (Table III). After a 45-min pretreatment of mice with 100 mg/kg Pargyline no labeled HVA or DOPAC could be found in the caudate nucleus 15 min after the intraventricular injection of ^{14}C-DA. As expected, the relative amounts of 3MT greatly increased. Thus O-methylation was apparently the only major metabolic route available for the catabolism of the labeled DA.

The considerable advantage of this TLC procedure lies in the fact that with a rapid and uncomplicated sample preparation, combinations of sixteen different ^{14}C-or ^{3}H-labeled catecholamines, their precursors and metabolites can be estimated directly without preparing derivatives.

TABLE III

In vivo METABOLISM OF ^{14}C-DA BY CONTROL AND MAO-INHIBITED MOUSE CAUDATE NUCLEUS

Reference standards	*Average % of recovered ^{14}C found in reference standards of interest, combined to total 100%*		
	Control (N = 1)	*Pargyline 100 mg/kg Pre-Rx 45 min (N = 2)*	
Origin	0	0	0
NE	0	3.8	0
DA	55.7	68.5	75.2
3MT	6.8	27.7	24.8
DOPAC	19.1	0	0
HVA	18.8	0	0

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