Journal of Chromatography, 254 (1983) 257–264
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,367

SENSITIVE AND SPECIFIC RADIOENZYMATIC ASSAY FOR NOREPINEPHRINE, EPINEPHRINE AND DOPAMINE BASED ON THE THIN-LAYER CHROMATOGRAPHIC SEPARATION OF THEIR Dns-O-METHYL DERIVATIVES

STEPHEN R. PHILIPS* and ALISON M. ROBSON
Psychiatric Research Division, University Hospital, Saskatoon, Saskatchewan S7N 0X0 (Canada)
(Received September 15th, 1982)

SUMMARY

A radioenzymatic assay is described in which norepinephrine, epinephrine and dopamine are converted to their tritiated 3-O-methyl derivatives by reaction with S-[methyl-^{3}H]adenosyl-L-methionine in the presence of catechol-O-methyltransferase. The methylated compounds are then reacted with Dns chloride, and the Dns derivatives are extracted into ethyl acetate, isolated by thin-layer chromatography and quantified by liquid scintillation spectrometry. The assay displays a high degree of specificity for each compound, due in large part to the chromatographic properties of the Dns derivatives. It is capable of measuring 2 pg of each catecholamine, and is linear to at least 5 ng. Approximately 50 samples can be assayed in 1.5 days.

INTRODUCTION

Engelman *et al.*[1] were the first to measure total catecholamines in biological specimens using a radioenzymatic assay in which a radiolabeled methyl group was transferred from S-[methyl-^{14}C]adenosyl-L-methionine ([^{14}C]SAM) to the catecholamines by the enzyme catecholamine-O-methyltransferase (E.C.2.1.1.6, COMT). Modifications to the original procedure have permitted measurements of norepinephrine (NE) and epinephrine (E)[2], the combined catecholamines and dopamine (DA)[3,4] or NE, E and DA[5,6] to be performed in a single sample. The sensitivity and specificity of the assay have been increased by using [^{3}H]SAM, which has a higher specific activity than [^{14}C]SAM, as the methyl donor[7], by separating the methylated derivatives by paper[5] or thin-layer[2,7–9] chromatography, or by oxidizing and separating the β-hydroxylated derivatives from those which contain no β-hydroxyl group[3,7]. In some cases, a liquid ion-exchange step has been introduced to extract the methylated derivatives from the enzyme reaction mixture; these are subsequently separated by thin-layer chromatography[10]. Gauchy *et al.*[11] have first isolated the catecholamines on microcolumns of alumina to remove inhibitors of the O-methylation reaction. We report here a relatively simple, yet very specific and sensitive radioenzymatic technique for measuring NE, E and DA as their highly fluorescent Dns-O-methylated deriva-

0021-9673/83/0000–0000/$03.00 © 1983 Elsevier Scientific Publishing Company

tives. Much of the specificity of the assay arises from the thin-layer chromatographic separation of the Dns compounds; only one extraction is required. As little as 2 pg of NE, E or DA can be determined.

EXPERIMENTAL

Materials

Dopamine · HCl, DL-norepinephrine · HCl, 3-methoxytyramine · HCl (3-MT), DL-normetanephrine · HCl (NMN), DL-metanephrine · HCl (MN), bovine serum albumin, Dns chloride, and ethyleneglycol-bis-(β-aminoethyl ether)N,N′-tetraacetic acid (EGTA) were purchased from Sigma (St. Louis, MO, U.S.A.). L-Epinephrine bitartrate was obtained from Calbiochem (San Diego, CA, U.S.A.), pargyline · HCl from Saber Labs. (Morton Grove, IL, U.S.A.), magnesium chloride (6 hydrate), L-ascorbic acid, and L-cysteine from J. T. Baker (Phillipsburg, NJ, U.S.A.), and tris-(hydroxymethyl)aminomethane (Tris) from Fisher Scientific (Fair Lawn, NJ, U.S.A.). S-[Methyl-^{3}H]adenosyl-L-methionine (9.6–13.7 Ci/mmol) and Omnifluor were purchased from New England Nuclear (Boston, MA, U.S.A.), while silica gel thin-layer chromatography plates (20 $\times$ 20 cm, 250 μm thickness) were supplied by Brinkmann Instruments (Canada) (Rexdale, Canada). All reagent-grade solvents were obtained from Fisher Scientific, except triethylamine (J. T. Baker) and toluene (Anachemia, Montreal, Canada). Except for acetone, which was distilled once before use, all chemicals and solvents were used without further purification. Water distilled from a glass apparatus in the presence of potassium permanganate was used to prepare all aqueous solutions.

Preparation of Catechol-O-methyltransferase

Livers from 3–4 male Wistar rats (body weight 250–300 g) were removed and chopped finely with a razor blade. To remove most blood from the tissue, the minced liver (35-40 g) was washed 8–10 times with 100–150-ml portions of isotonic saline. After freezing briefly in a vessel cooled in dry ice–acetone, the tissue was thawed, homogenized in 4 volumes (w/v) of 1.15% KCl, and centrifuged at 10,000 *g* for 10 min. The supernatant was carefully removed and centrifuged at 78,000 *g* for 1 h. An enzyme fraction was isolated from the second supernatant by ammonium sulfate fractionation according to the procedure of Coyle and Henry[3]. The 30–55% precipitate was dissolved in 15–20 ml of 1 m*M* sodium phosphate buffer, pH 7.0, then dialyzed at 4°C for 20 h against 3.5 l of the same buffer. The buffer was changed twice during dialysis. The final product was centrifuged at 10,000 *g* for 10 min to remove precipitates, then stored in 1.5-ml portions at −20°C until used. The enzyme was stable for at least one month.

Preparation of samples

Brain tissue samples (hypothalamus, cerebellum, medulla oblongata and striatum, dissected as described by Glowinski and Iversen[12]), were homogenized in 20–500 volumes of ice-cold 0.6 *N* perchloric acid (PCA). The homogenates were centrifuged at 10,000 *g* for 15 min at 4°C and the supernatants were removed for analysis. The samples could be assayed immediately or stored at −20°C. Standard solutions of the catecholamines (1 mg/ml) were prepared in 0.01 *N* hydrochloric acid containing 1 mg/ml ascorbic acid. They were stored at −20°C and were diluted as required for each assay.

Assay for norepinephrine and epinephrine

Duplicate samples of homogenized tissue supernatant (50 μl) were placed in cold 15-ml centrifuge tubes. To one sample was added 25 μl of distilled water–0.6 *N* PCA (5:1, v/v) containing a known amount of the catecholamines to be determined. The volume of each sample was then adjusted to 300 μl with distilled water. Reagent blanks, consisting of 300 μl of distilled water–0.6 *N* PCA (5:1, v/v) and standard samples containing the catecholamines to be assayed (usually 50 pg) in 300 μl of distilled water–0.6 *N* PCA (5:1, v/v) were run in triplicate with each assay.

The enzyme reaction was initiated by adding to each sample 100 μl of a cold freshly-prepared enzyme mixture containing (final concentrations in parentheses) 5 μl aqueous pargyline·HCl (0.6 m*M*), 10 μl aqueous EGTA (1.25 m*M*), 50 μl Tris acetate buffer, pH 9.1 (350 m*M*) containing $MgCl_2$ (40 m*M*) and ascorbic acid (0.1 m*M*), 5 μl (2.5 μCi) [^{3}H]SAM (0.6 μ*M*), 25 μl COMT solution (approximately 350 μg protein), 5 μl distilled water–0.6 *N* PCA (5:1, v/v) and 1 mg bovine serum albumin (0.25%, w/v). The samples were kept at 0°C until all additions had been completed, then mixed for 10 sec on a vortex mixer, transferred to a water bath, and incubated for 1 h at 37°C. The enzyme reaction was stopped by cooling the tubes to 0°C and adding 300 μl of 0.5 *M* sodium borate, pH 10, containing 3 μg of NMN and MN as carriers.

Dns derivatives of the methylated catecholamines were prepared by saturating the enzyme reaction mixture with $NaHCO_3$ and adding 1 ml of Dns chloride reagent (4 mg/ml acetone). The reaction was allowed to proceed overnight at room temperature. Because some Dns compounds are known to be subject to photochemical degradation on silica gel plates[13,14], all chromatographic procedures involving these substances were performed in subdued light. Dns-NMN and Dns-MN were extracted from the enzyme reaction solution by vigorous mixing for 1 min with two 1.5-ml portions of water-saturated ethyl acetate. The extracts were combined and dried under a stream of nitrogen, then redissolved in a few drops of methanol, and applied to a silica gel thin layer plate. Extracts from six samples, as well as the standard Dns-amines could be applied to each plate. Dns-NMN and Dns-MN were purified by successive separations on two thin-layer chromatograms using the solvent systems toluene–triethylamine–methanol (100:15:10, v/v/v) and chloroform–*n*-butyl acetate–ethanol (100:40:10, v/v/v). The separated fluorescent zones, visualized briefly under UV light, were removed from the second chromatogram and placed in vials containing 1 ml methanol and 10 ml Omnifluor liquid scintillation cocktail (4 g/l toluene). Radioactivity was determined by liquid scintillation counting.

Assay for dopamine

The assay is identical to that described for NE and E, except that (1) in the enzyme reaction mixture, $MgCl_2$ was present at a final concentration of 20 m*M*, and cysteine (final concentration 1 m*M*) was used as antioxidant, and (2) the enzyme reaction was stopped by adding 300 μl of 0.5 *M* sodium borate, pH 11, containing 3 μg of 3-MT as carrier.

RESULTS

During development of the assay, several parameters of the enzyme reaction

and subsequent purification procedures were examined to establish the conditions required for maximum sensitivity. For each catecholamine sensitivity was optimized with respect to the pH of the enzyme reaction when the pH of the Tris acetate buffer was 9.1. The final pH of the enzyme reaction mixture under these conditions, 8.3, was near the optimum reported for enzyme activity[15,16]. Maximum sensitivity was obtained in the NE and DA assays when 0.5 *M* sodium borate, pH 10 or 11 respectively, was used to stop the enzyme reaction.

COMT activity is known to depend on the presence of magnesium ions[15], but estimates of the ion concentration required for maximum activity vary widely and appear to depend upon the properties of the sample being assayed[7,15–17]. Blank and standard samples (50 pg) of NE and DA were assayed using enzyme reaction mixtures containing 0–60 m*M* magnesium. The radioactivity recovered in the Dns-NMN and Dns-3-MT zones, and the sample:blank ratio (sensitivity) were greatest when NE and DA were determined in the presence of 40 and 20 m*M* magnesium, respectively. No enzyme activity was present in the absence of magnesium.

EGTA was routinely added to the enzyme reaction mixture to a final concentration of 1.25 m*M* to ensure complete chelation of calcium ions present in the tissue samples, since COMT has been reported to be inhibited by calcium[16]. Bovine serum albumin, added in small amounts (0.25%, w/v) to the COMT reaction mixture, increased the reproducibility, linearity and sensitivity of the assay, presumably by preventing interaction of the enzyme with the glass reaction tubes[16].

Dns-methylated catecholamines were extracted into ethyl acetate to separate the tritated COMT reaction products from unreacted [^{3}H]SAM, most of which remained in the aqueous phase[8]. The extraction also served to transfer the Dns compounds from the aqueous enzyme reaction mixture to a volatile solvent, thus enabling the sample volume to be reduced rapidly for application to thin layer plates. The extracts were dried under nitrogen, redissolved in 4–5 drops of methanol and applied as narrow bands (4–5 mm wide) to a silica gel plate. On the developed chromatogram, Dns-MN appeared as a narrow (5–6 mm wide) fluorescent band clearly separated from Dns-NMN and Dns-3-MT (see Table I for R_F values). If NE, E and DA were determined simultaneously in the same sample, Dns-MN and Dns-3-MT were not completely separated by the first solvent system, but could be clearly and completely separated by the second solvent system (Table I).

TABLE I

SEPARATION OF Dns-NORMETANEPHRINE, Dns-METANEPHRINE AND Dns-3-METHOXYTYRAMINE BY THIN-LAYER CHROMATOGRAPHY

Solvent 1: toluene–triethylamine–methanol (100:15:10, v/v/v). Solvent 2: chloroform–*n*-butyl acetate–ethanol (100:40:10, v/v/v).

Compound	*R_F value*	
	Solvent 1	*Solvent 2*
Dns-normetanephrine	0.45	0.48
Dns-metanephrine	0.59	0.57
Dns-3-methoxytyramine	0.57	0.69

The specificity of the assays was determined by reacting with COMT one nanogram of a variety of compounds having molecular structures similar to those of the catecholamines. The amounts of radioactivity detected in the Dns-NMN, Dns-MN and Dns-3-MT zones were compared to those observed in these zones when equal amounts (1 ng) of NE, E and DA, respectively, were assayed. Of the substances examined, only L-dopa and 6-hydroxydopamine interfered to an appreciable extent (3.74 % and 4.26 %, respectively) with the determination of DA, while DA cross-over occurred to a slight extent (0.66 %) in the assay for E (Table II). The high degree of specificity attained can be attributed in large part to the chromatographic characteristics of the Dns-methylated catecholamines. The narrow bands into which the Dns compounds separate ensure that these substances are clearly separated from each other and from other possible contaminating substances.

The assay sensitivity, defined as the amount of a substance which will double the blank dpm, was 2–4 pg for NE, E and DA. Minor differences in the procedures for DA and for NE and E, incorporated to obtain maximum sensitivity in the determination of each compound, necessitated that DA be determined separately from NE

TABLE II

ASSAY SPECIFICITY

One nanogram of each compound was assayed in triplicate. The radioactivity recovered in the Dns-normetanephrine, -metanephrine and -3-methoxytyramine zones is expressed as a percentage of that recovered in these zones when 1 ng of norepinephrine, epinephrine and dopamine, respectively, was assayed.

Compound	*Dns-normetanephrine*	*Dns-metanephrine*	*Dns-3-methoxytyramine*
Norepinephrine	100.00	0.11	0.02
Epinephrine	0.01	100.00	0.21
Dopamine	0.04	0.66	100.00
Normetanephrine	0.16	0.01	0.08
Metanephrine	0.10	0.29	0.09
3-Methoxytyramine	0.04	0.04	0.20
L-Dopa	0.05	0.11	3.74
α-Methyldopa	0.03	0.16	0.08
3-Methoxy-4-hydroxyphenylglycol	0.11	0.16	0.03
3,4-Dihydroxyphenylglycol	0.10	0.12	0.00
3,4-Dihydroxyphenylacetic acid	0.03	0.09	0.04
Homovanillic acid	0.06	0.07	0.00
Dihydroxymandelic acid	0.08	0.18	0.11
m-Tyramine	0.03	0.11	0.07
p-Tyramine	0.08	0.11	0.04
p-Octopamine	0.06	0.00	0.06
p-Synephrine	0.06	0.18	0.02
Phenylethylamine	0.02	0.13	0.01
Phenylethanolamine	0.00	0.07	0.03
Tryptamine	0.00	0.01	0.05
5-Hydroxytryptamine	0.01	0.14	0.02
5-Hydroxyindoleacetic acid	0.03	0.05	0.03
Amphetamine	0.01	0.02	0.07
α-Methyl-*p*-tyrosine	0.08	0.09	0.02
6-Hydroxydopamine	0.01	0.20	4.26
3,4-Dihydroxybenzoic acid	0.02	0.24	0.04

and E. However, the three compounds could be determined in a single sample, according to the procedure described for NE and E, with only a 2–3-fold reduction in DA sensitivity. The assay for each catecholamine was linear from the limit of sensitivity to 5–10 ng.

Intra-assay reproducibility was assessed by assaying six 50-pg samples of each standard catecholamine. The coefficient of variation, determined from the dpm recovered in the respective Dns-methylated derivatives, was 6.5% or less for each amine. The inter-assay variability, determined by assaying 50 pg samples of each standard catecholamine on ten different occasions, was 8.6% or less for each amine. When the catecholamine content of a single tissue homogenate was measured on six separate occasions, the coefficients of variation for NE, E and DA were 7.3%, 9.7% and 6.5%, respectively.

Catecholamine concentrations in the hypothalamus, cerebellum, medulla oblongata and striatum of the rat are listed in Table III.

TABLE III

NOREPINEPHRINE, EPINEPHRINE AND DOPAMINE CONCENTRATIONS IN RAT BRAIN REGIONS

Data are expressed as ng/g fresh tissue ± S.E.M.; n = 5–10. Epinephrine data have been corrected for dopamine cross-over.

Brain region	*Norepinephrine*	*Epinephrine*	*Dopamine*
Hypothalamus	2430 ± 80	70 ± 9	451 ± 17
Cerebellum	255 ± 5	5 ± 2	9 ± 2
Medulla Oblongata	790 ± 58	22 ± 5	59 ± 3
Striatum	82 ± 9	67 ± 23	12,780 ± 400

DISCUSSION

Radioenzymatic assays have been widely used to determine catecholamines in tissues and body fluids[18]. We required an assay capable of measuring less than 10 pg of NE and DA in perfusates collected from a push-pull cannula inserted into specific areas of the rat brain. Although some published assays appeared to be capable of such sensitivity[9,10,17], all required the enzyme reaction products to be separated by extensive extraction and chromatographic procedures. As Robertson *et al.*[19] had shown earlier, the Dns derivative of O-methylated NE could be readily prepared; it seemed appropriate to extend their findings to O-methylated E and DA. In most assays, the O-methylated enzyme reaction products are extracted from the reaction mixture prior to further purification [*e.g.*[3,7]]. Direct Dns derivatization of the enzyme reaction mixture and subsequent separation of Dns-NMN, Dns-MN and Dns-3-MT as described here, however, eliminate the need for most time-consuming extractions, and permit about 50 samples to be assayed in 1.5 days. Attempts to increase sensitivity by extracting the methylated catecholamines prior to Dns derivatization were unsuccessful; in no instance was the sensitivity obtained by including an organic extraction by a variety of solvents greater than that obtained by Dns derivatizing the enzyme reaction mixture directly.

Much of the assay specificity can be attributed to the chromatographic proper-

ties of the Dns-methylated catecholamines. These easily prepared derivatives can all be readily extracted into ethyl acetate, thus allowing simultaneous determination of NE, E and DA. The volatility of the organic solvent allows the extract to be reduced rapidly to a volume suitable for application to a thin-layer chromatogram. The intense fluorescence of the Dns compounds under UV light enables them to be identified readily. Because they separate into narrow bands, distinct and complete separation of the Dns-methylated catecholamines from each other and from other potentially interfering substances is ensured. Of the compounds tested, only L-dopa and 6-hydroxydopamine interfered with the determination of DA. Small amounts of DA, previously shown to be formed from L-dopa by the decarboxylase present in partially purified COMT preparations[8], likely account for the apparent cross-over of L-dopa in the present assay, while Dns-methylated 6-hydroxydopamine may not be completely separated from Dns-3-MT in the solvent systems used. Although none of the substances examined interfered significantly with the NE assay, DA contributed to the extent of approximately 0.7% to the activity recovered in the Dns-MN zone. Hence, in samples containing significant amounts of DA, corrections in E estimations were required to eliminate the contribution to the activity in the Dns-MN zone due to DA. In instances in which the concentration of DA greatly exceeds that of E, for example in the caudate nucleus, E estimations may be subject to substantial error.

Catecholamines in brain regions were determined by assaying duplicate samples of tissue homogenate, one of which was supplemented with known quantities of the catecholamines. By adding internal standards to each sample, rather than comparing each sample to a standard curve, errors arising from sample differences may be reduced. Striatal DA levels reported here are in excellent agreement with those recently obtained using mass fragmentographic[20] and radioenzymatic[21] techniques. NE concentrations in the striatum are lower than those reported earlier[21,22], but in the medulla oblongata and cerebellum, our results agree closely with those obtained by Nomura *et al.*[23] using a spectrofluorometric assay. Because of differences in the tissue regions assayed, comparisons are difficult between E concentrations reported here and those reported earlier. However, E appears to be present in considerably smaller quantities in the whole cerebellum and medulla oblongata (Table III) than in the nuclei of these regions[5].

Although we have measured catecholamine concentrations only in large brain regions of the rat in order to permit comparison with values obtained by other techniques, the assay is sufficiently sensitive to allow determinations to be made in very small tissue samples such as individual nuclei, and has recently been used to determine the unstimulated and amphetamine-stimulated release rates of endogenous NE and DA from the rat brain *in vivo*[24]. It is likely that other compounds which undergo the COMT and Dns derivatization reactions could also be measured in a similar manner, although some adjustment to the thin-layer chromatographic solvents may be required to separate specific substances from interfering materials.

ACKNOWLEDGEMENTS

We thank Dr. A. A. Boulton for critical reading of the manuscript, Mr. H. Miyashita and Mr. R. C. Mag-Atas for expert technical assistance, and the Medical Research Council and Saskatchewan Health, Province of Saskatchewan for continuing financial support.

REFERENCES

1 K. Engelman, B. Portnoy and W. Lovenberg, *Amer. J. Med. Sci.*, 255 (1968) 259.
2 K. Engelman and B. Portnoy, *Circ. Res.*, 26 (1970) 53.
3 J. T. Coyle and D. Henry, *J. Neurochem.*, 21 (1973) 61.
4 A. C. Cuello, R. Hiley and L. L. Iversen, *J. Neurochem.*, 21 (1973) 1337.
5 J. Van der Gugten, M. Palkovits, H. L. J. M. Wijnen and D. H. G. Versteeg, *Brain Res.*, 107 (1976) 171.
6 V. K. Weise and I. J. Kopin, *Life Sci.*, 19 (1976) 1673.
7 P. G. Passon and J. D. Peuler, *Anal. Biochem.*, 51 (1973) 618.
8 N. Ben-Jonathan and J. C. Porter, *Endocrinology*, 98 (1976) 1497.
9 J. D. Peuler and G. A. Johnson, *Life Sci.*, 21 (1977) 625.
10 M. Da Prada and G. Zurcher, *Life Sci.*, 19 (1976) 1161.
11 C. Gauchy, J. P. Tassin, J. Glowinski and A. Cheramy, *J. Neurochem.*, 26 (1976) 471.
12 J. Glowinski and L. L. Iversen, *J. Neurochem.*, 13 (1966) 655.
13 N. Seiler, *Methods Biochem. Anal.*, 18 (1970) 259.
14 M. I. Pouchan and E. J. Passeron, *Anal. Biochem.*, 63 (1975) 585.
15 J. Axelrod and R. Tomchick, *J. Biol. Chem.*, 233 (1958) 702.
16 R. M. Weinshilboum and F. A. Raymond, *Biochem. Pharmacol.*, 25 (1976) 573.
17 C. F. Saller and M. J. Zigmond, *Life Sci.*, 23 (1978) 1117.
18 S. R. Philips, in S. Federoff and L. Hertz (Editors), *Advances in Cellular Neurobiology*, Vol. 2, Academic Press, New York, 1981, p. 355.
19 H. A. Robertson, J.-C. David and T. J. Danielson, *J. Neurochem.*, 29 (1977) 1137.
20 C. D. Kilts, J. J. Vrbanac, D. E. Rickert and R. H. Rech, *J. Neurochem.*, 28 (1977) 465.
21 N. S. Sharpless and L. L. Brown, *Brain Res.*, 140 (1978) 171.
22 M. Brownstein, J. M. Saavedra and M. Palkovits, *Brain Res.*, 79 (1974) 431.
23 Y. Nomura, F. Naitoh and T. Segawa, *Brain Res.*, 101 (1976) 305.
24 S. R. Philips, A. M. Robson and A. A. Boulton, *J. Neurochem.*, 38 (1982) 1106.