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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF NOREPINEPHRINE AND α-METHYLNOREPINEPHRINE IN MICRODISSECTED NORADRENERGIC CELL GROUPS OF THE RABBIT BRAIN

C.J. ODDIE*, G.K. MILL, P.J. SCOTT and A. BOBIK

Clinical Research Unit, Alfred Hospital, and Baker Medical Research Institute, Prahran, Victoria 3181 (Australia)

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SUMMARY

A high-performance liquid chromatographic method with electrochemical detection has been developed to measure norepinephrine (NE) and α-methylnorepinephrine (α-MeNE) in microdissected noradrenergic cell groups of the rabbit brain stem. The method is shown to be specific, accurate and precise and has a sensitivity of 0.5 pmol per sample. The method has been applied to the measurement of NE and α-MeNE levels in the A1, A2, A5, A6 and A7 noradrenergic cell groups after administration of the antihypertensive drug α-methyldopa (α-MD), so that the sites of action of α-MD within the central nervous system may be localised.

INTRODUCTION

It is presently thought that the antihypertensive agent L-α-methyldopa (α-MD) produces a large component of its effect through its action on monoamine neurons within the central nervous system [1—3]. Microinjections of α-methylnorepinephrine (α-MeNE), a metabolite of α-MD, into norepinephrine (NE)-containing areas such as the anterior hypothalamic—preoptic region or the nucleus tractus solitarius of the brain induce falls in blood pressure in anaesthetized rats [4, 5]. Analysis of catecholamines in these regions by radioenzymatic techniques has shown that α-MeNE concentrations rise rapidly after administration of α-MD whilst NE levels decline [6, 7]. Despite such studies, the precise sites of action of α-MD within the central nervous system remain to be identified [8]. This is due in part to the very limited information currently

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available on the distribution and metabolism of the drug within the brain. Radioenzymatic methods utilising the enzyme catechol-O-methyltransferase and [^{3}H]S-adenosyl-L-methionine to measure NE and α-MeNE within noradrenergic neurons of the brain are not suitable for detailed distribution studies because they are relatively difficult to perform and involve time-consuming paper chromatographic separations of the [^{3}H]methylated catecholamines [6].

The present paper describes a high-performance liquid chromatographic (HPLC) method for measuring NE and α-MeNE in microdissected noradrenergic cell groups of the brain stem (A1, A2, A5, A6, A7) [9]. The method has been used to study the distribution and metabolism of α-MD and its effect on catecholamines in discrete areas of the brain.

EXPERIMENTAL

Reagents

Stock solutions (1 mg/ml) of norepinephrine bitartrate (Sigma, St. Louis, MO, U.S.A.), α-methylnorepinephrine (Sterling-Winthrop, Rensselaer, NY, U.S.A.) and dihydroxybenzylamine (DHBA, Sigma) were prepared in 0.1 *M* hydrochloric acid. For each assay run, working standard solutions were prepared by diluting the stock solutions with 0.01 *M* hydrochloric acid (NE and α-MeNE) or 5% trichloroacetic acid (TCA) (DHBA). Alumina (Merck, Darmstadt, F.R.G.) was activated by the method of Anton and Sayre [10]. Water for HPLC was redistilled from alkaline potassium permanganate. All other reagents were either of HPLC or analytical grade.

Microdissection of noradrenergic cell groups

The brain was removed from rabbits immediately after an overdose of pentobarbitone (May and Baker, West Footscray, Australia) and suspended in air at −30°C until frozen. The forebrain was removed and the brain stem and midbrain stored overnight at −80°C. The following day, frozen portions of the brain were mounted in a cryostat (International Equipment/Damon, Needham, MA, U.S.A.) at −14°C and 200-μm serial, coronal sections cut and transferred to slides. Five noradrenergic cell groups — A1, A2, A5, A6 and A7 [9] (Fig. 1) — were dissected from the frozen sections as described by Palkovits [11] using a 1.3 mm I.D. square-cut needle. The precise locations of the respective cell groups were obtained from histological maps of NE-containing cell groups of the rabbit brain prepared according to Blessing et al. [12]. After dissecting the respective areas, each section was fixed with 10% formalin and stained with cresyl violet to confirm major landmarks and the noradrenergic cell group punch positions.

The tissue biopsies obtained from the brain sections for each 1 mm rostrocaudal depth (i.e. 5 × 200 μm sections), right and left side, were pooled as shown in Fig. 1 and stored in 1.5-ml microtubes (Eppendorf, F.R.G.) at −80°C until analysed.

Tissue homogenisation

Brain biopsies were homogenised in 100 μl of ice cold 5% TCA containing

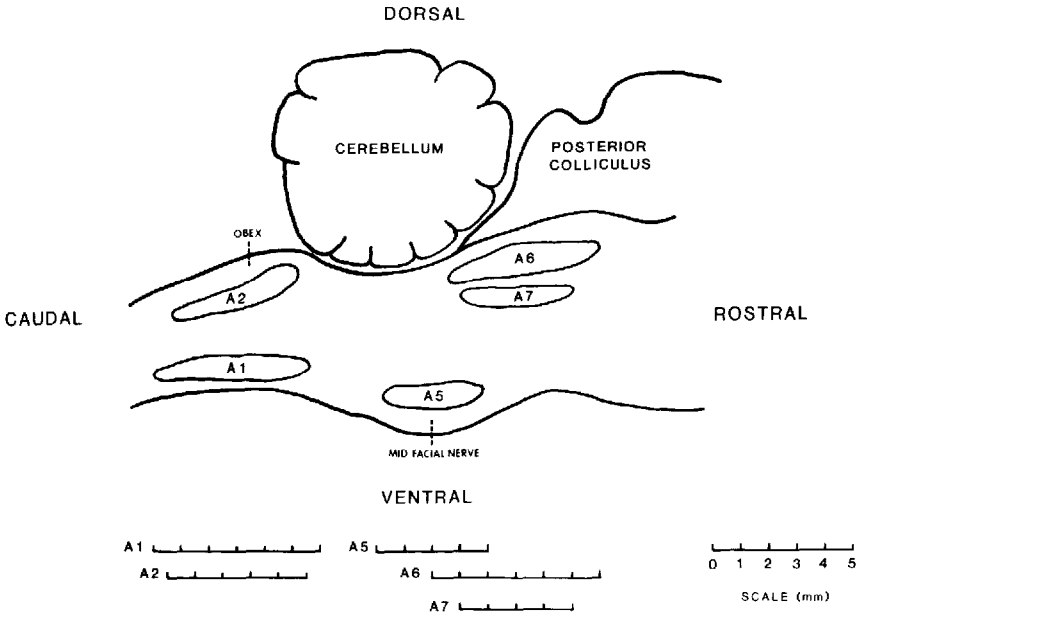

Fig. 1. Diagrammatic representation of the rabbit brain showing the location of the noradrenergic cell groups (A1, A2, A5, A6, A7) and their microdissection.

29 pmol DHBA in situ in the microtubes using a motorised PTFE pestle. The pestle was rinsed three times with 100 µl of 5% TCA and the rinsings were added to the original homogenate. The samples were centrifuged at 9980 *g* (Zentrifuge 5412, Eppendorf) for 10 min at 4°C. The resultant supernatant was used for catecholamine analysis whilst the pellet was assayed for protein content [13] after digestion in 1 *M* sodium hydroxide for 18 h at 37°C. Bovine serum albumin was used as the protein standard.

Catecholamine analysis

Norepinephrine and α-methylnorepinephrine. Homogenate supernatant (300 µl) or 5% TCA containing 0—95 pmol of standard NE and α-MeNE and 29 pmol of DHBA were diluted to 2.0 ml with 5% TCA on ice. Buffer (1 *M* Tris—HCl, 2% EDTA, pH 8.6, 3.5 ml) and activated alumina (100 mg) were added and the samples shaken for 3 min. After brief centrifugation, the liquid was aspirated and the alumina washed with distilled water (3 × 10 ml). Catecholamines were eluted from the alumina with 0.2 *M* perchloric acid (200 µl) using a small filtration apparatus.

Catecholamines in the eluates were quantified by injection on to a Model 5000 liquid chromatograph fitted with a 100-µl universal loop injector (Varian Assoc., Palo Alto, CA, U.S.A.) and a Spherisorb 5-µm ODS-1 column (25 cm × 4.6 mm I.D., Laboratory Data Control, Riviera Beach, FL, U.S.A.). The mobile phase was 0.01 *M* perchloric acid at a flow-rate of 1 ml/min. Catecholamines were detected electrochemically (Model LC-4A, Bioanalytical Systems, West Lafayette, IN, U.S.A.) using a glassy carbon electrode. Oxidation potential was set at 0.70 V versus an Ag/AgCl reference electrode. Detector response was quantified using a computing integrator (Model 308, Laboratory Data Control).

Retention times for NE, α-MeNE and DHBA were 5.4, 7.0 and 8.8 min, respectively.

The specificity of the assay for NE and α-MeNE was evaluated by comparing the effect of changes in oxidation voltage on detector response between standard NE and α-MeNE and those extracted from tissue homogenates. The accuracy of the assay was examined by adding known amounts of NE and α-MeNE to 5% TCA and tissue homogenate and processing the samples through the assay. Intra-assay precision was determined by replicate analysis of a pool of brain biopsy homogenates.

α-Methyldopa. α-MD was measured in the brain biopsies by diluting 50 μl of homogenate supernatant or α-MD standard (Merck Sharp and Dohme, South Granville, Australia) (0—330 pmol), prepared in non-α-MD-treated rabbit brain homogenate supernatant, with 150 μl of 0.05 *M* disodium hydrogen phosphate on ice.

α-MD was quantified by HPLC with electrochemical detection (ED) using a Spherisorb 5-μm ODS-2 column (15 cm × 4.6 mm I.D., Phase Separations, Queensferry, U.K.). The mobile phase was 0.1 *M* sodium dihydrogen phosphate, 2 m*M* sodium heptane sulphonate, 0.001% disodium EDTA, pH 4.7, at a flow-rate of 1.5 ml/min. Oxidation protential was set at 0.80 V versus Ag/AgCl. Retention time for α-MD was 5.3 min.

Fluorescence histochemistry

In order to further validate the catecholamine assay procedure, the distribution of NE within the five noradrenergic cell groups was correlated with the number of NE-containing cells in the respective areas. The number of NE-containing cells was quantitated by the formaldehyde—glutaraldehyde (Faglu) fluorescence technique of Furness et al. [14]. Rabbits were overdosed with pentobarbitone before perfusion with the formaldehyde—glutaraldehyde mixture. Serial, coronal sections (30 μm) were cut using a vibratome (Oxford Instruments, U.S.A.) and the cells exhibiting catecholamine fluorescence counted using a Leitz Orthoplan microscope with UV epi-illumination.

RESULTS

Catecholamine estimation

Typical chromatograms of catecholamine standards and extracts prepared from the microdissected areas of the brain stem are shown in Fig. 2. NE, α-MeNE and the internal standard, DHBA, are well resolved from each other, from other peaks and from the solvent front. The peaks identified as NE and α-MeNE (retention times 5.4 and 7.0 min, respectively) in the extracts gave identical detector response as authentic standards when the oxidation potential was varied between 0.6 and 0.8 V. In the chromatographic system used, epinephrine has a similar retention time to DHBA (8.9 min); however, epinephrine could not be detected in any of the noradrenergic cell group areas. α-MD and its metabolite, α-methyldopamine, chromatographed with retention times of 28 and 31 min, respectively.

The calibration curves for NE and α-MeNE added to TCA and processed through the assay were linear and reproducible over the range 0—95 pmol.

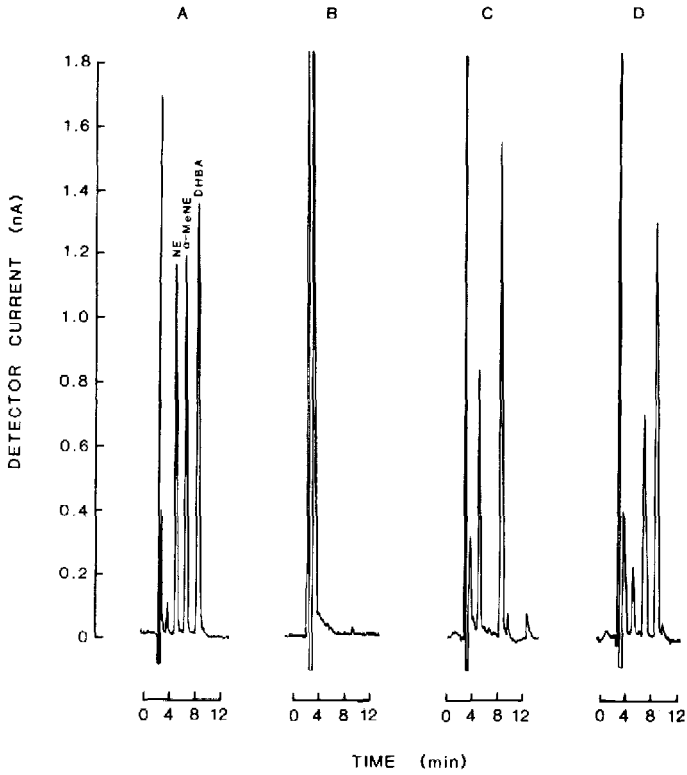

Fig. 2. Chromatographic traces of standards and microdissected tissue homogenate extracts. (A) Standard NE (3 pmol), α-MeNE (3 pmol) and internal standard (DHBA, 3 pmol); (B) assay blank; (C) tissue homogenate from A1 cell group containing 21.0 pmol NE per homogenate; (D) tissue homogenate from A5 cell group following α-MD administration (600 μg/kg intracisternally, 3 h) and containing 4.02 pmol NE per homogenate and 14.3 pmol α-MeNE per homogenate.

When standard NE or α-MeNE was added to tissue homogenate and carried through the assay procedure, the resultant regression lines (NE, $y = 0.323x + 0.734$; α-MeNE, $y = 0.257x + 0.947$) were parallel to those for TCA (NE, $y = 0.312x - 0.0071$; α-MeNE, $y = 0.256x - 0.017$). Recoveries of NE, α-

TABLE I

INTRA-ASSAY PRECISION OF THE METHOD FOR NE AND α-MeNE

Sample No.	NE			α-MeNE		
	Concentration (mean ± S.D.) (pmol/homogenate)	*n*	C.V.* (%)	Concentration (mean ± S.D.) (pmol/homogenate)	*n*	C.V.* (%)
1	6.80 ± 0.183	4	2.7	18.2 ± 0.213	5	1.2
2	15.7 ± 0.290	6	1.8	26.4 ± 0.672	6	2.5
3	41.8 ± 1.41	6	3.4	52.5 ± 2.22	6	4.2

*C.V. = coefficient of variation.

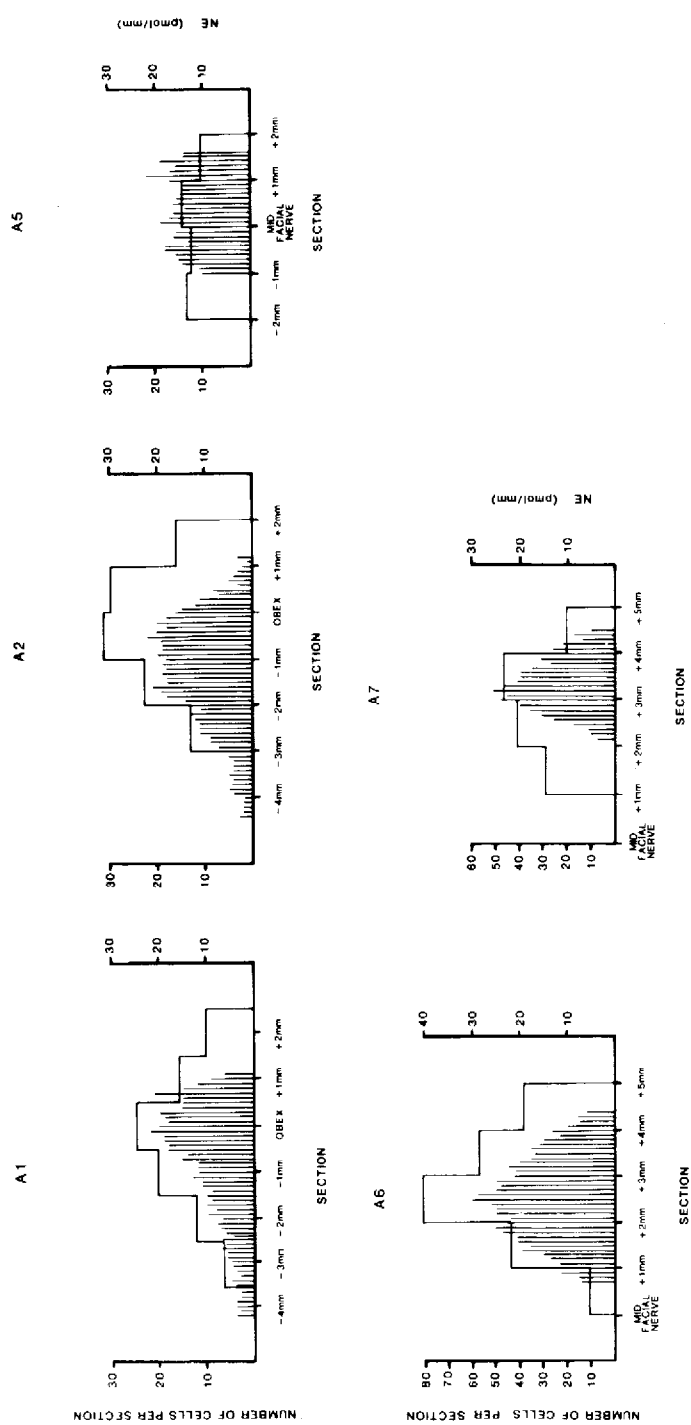

Fig. 3. NE content of microdissected noradrenergic cell groups of the rabbit brain stem (A1, A2, A5, A6, A7) diagrammatically superimposed on the distribution of NE-containing nuclei as determined by Faglu fluorescence. Each diagram reads caudal to rostral from left to right.

MeNE and DHBA averaged 49, 54 and 53%, respectively. Determination of the intra-assay precision for NE and α-MeNE was assessed by replicate analysis of pooled microdissected brain homogenates and yielded coefficients of variation of 1.2—4.2% (Table I). Detection limits for NE and α-MeNE were 0.5 pmol per homogenate.

Relation between NE content and noradrenergic cell number

In the A1, A5 and A6 areas, there was close correlation between the NE content of the cell group and the number of catecholamine-containing cells (Fig. 3). In the A2 cell group, NE content correlated well with cell number in sections caudal to the obex (—1 to —4 mm), but deviated in the rostral sections (+1 to +2 mm). The deviation in these sections is due to a predominance of NE-containing nerve terminals and dendrites. In the A7 cell group, +1 to +2 mm from the mid facial nerve, the high NE content relative to the small number of catecholamine-containing cells is also due to a high density of NE-containing terminals. In the more rostral sections of this cell group, NE content correlated with cell number.

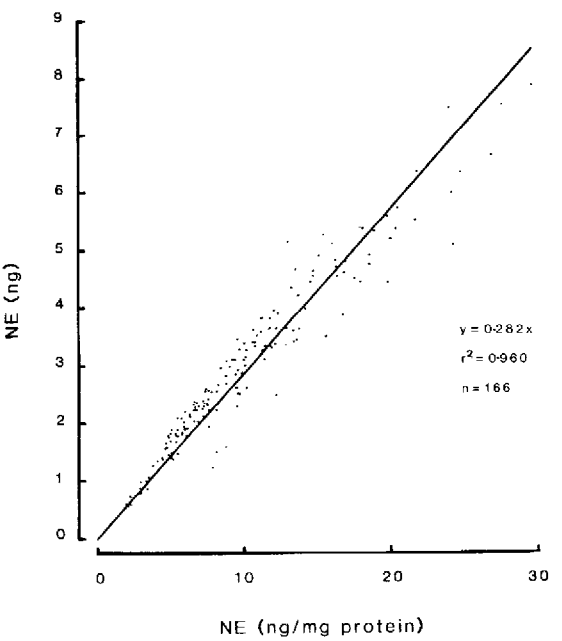

Fig. 4. Correlation of NE content of microdissected cell group (A1—A7) samples (ng per sample) and the NE content expressed as ng per mg protein in seven control rabbits.

The microdissection of the cell groups was highly reproducible between rabbits. This was determined by correlating the NE content in each sample (ng per sample) with the NE content related to the protein concentration of the sample (ng per mg protein). The correlation coefficient was 0.960 (Fig. 4). The protein concentrations in the A1 to A7 cell group areas averaged 311 ± 3, 268 ± 5, 324 ± 4, 269 ± 3 and 310 ± 3 (mean ± standard error of the mean) μg per sample, respectively.

Catecholamine content after α-MD administration

The histological and analytical techniques were used to measure the NE and α-MeNE content of noradrenergic cell groups of the rabbit brain following intravenous or intracisternal α-MD administration. In the A1 cell group, intravenous α-MD (50 mg/kg) greatly increased total catecholamine (NE plus α-MeNE) content (Table II). The largest increases (82—135%) were seen at the obex and areas rostral (+1 and +2 mm) to the obex. The size of these increases appeared to be related to the effect of α-MD on NE content. In the areas above, NE was depleted by 50—63% after the α-MD dose. In contrast, in the areas caudal (—2 and —3 mm) to the obex, NE content was reduced by 84—93% and total catecholamine content was increased by only 32 and 38% over control levels. The distribution of α-MD did not correlate with either the distribution of NE-containing cells or NE content in the cell group. In contrast, the distribution of the metabolite, α-MeNE, did correlate with the distribution of both NE-containing cells and NE content. The metabolite, α-methyldopamine, was found in brain biopsy homogenates in the 1—10 pmol range, but was not specifically measured in this study.

TABLE II

CONCENTRATIONS OF NE, α-MeNE AND α-MD WITHIN THE A1 CELL GROUP 3 h AFTER ADMINISTRATION OF α-MD (50 mg/kg INTRAVENOUSLY) TO FOUR RABBITS

Values are mean ± standard error of the mean. For control NE levels, $n = 7$.

Section	Concentration (pmol/mg of protein)				
	Control NE level	After α-MD administration			
		NE	α-MeNE	NE + α-MeNE	α-MD
—3 mm	21.4 ± 2.4	1.5 ± 0.92	26.7 ± 2.8	28.2 ± 3.7	223 ± 38
—2 mm	37.7 ± 2.7	6.0 ± 2.4	46.1 ± 5.4	52.1 ± 7.8	160 ± 18
—1 mm	61.1 ± 4.4	23.5 ± 3.2	83.6 ± 5.8	107 ± 9.0	145 ± 23
Obex	77.3 ± 4.2	37.8 ± 8.2	103 ± 12.1	141 ± 20.3	125 ± 18
+1 mm	49.0 ± 3.4	24.3 ± 4.8	90.2 ± 11.8	115 ± 16.6	136 ± 18
+2 mm	31.9 ± 2.5	11.9 ± 3.5	59.3 ± 2.1	71.2 ± 5.6	138 ± 14

DISCUSSION

The present study has optimised an HPLC—ED assay for measuring NE and α-MeNE in microdissected rabbit brain. The assay shows specificity, accuracy and precision. It has a lower limit of sensitivity of 0.5 pmol per sample which is comparable to that of a radioenzymatic assay for brain catecholamines [6] and to the HPLC—ED assay for plasma catecholamines previously reported from this laboratory [15].

The simplified HPLC—ED procedure was possible because levels of α-MD in the brain biopsies were an order of magnitude lower than those found in plasma following α-MD administration [15]. Initial separation of catecholamines and α-MD using a cation-exchange resin was therefore not necessary and the α-MD present chromatographed on HPLC with a comparatively long

retention time. Perchloric acid was used as the mobile phase for the present HPLC—ED assay for two reasons. Firstly, shorter injection times than with the phosphate mobile phase were possible and secondly, it was not necessary to separate the internal standard (DHBA) from endogenous epinephrine because the latter catecholamine was not detectable in the noradrenergic cell group biopsies. In species such as the rat, in which appreciable levels of epinephrine are found, alternative internal standards, for example *n*-ethylnoradrenaline, could be used.

The HPLC—ED procedure described here has several advantages over previously reported radioenzymatic techniques. It is a comparatively simpler technique and does not involve time-consuming chromatographic separations and expensive radiochemicals. Thus a larger number of brain biopsy samples may be studied by HPLC—ED to determine the distribution and turnover of catecholamines in discrete areas of the brain.

A quantitative histofluorometric technique has recently been developed to measure catecholamines in the rat brain [16]. The procedure may be used to measure catecholamine turnover rates following treatment with α-methyl-*p*-tyrosine, but it is not suitable for studying the effects of α-MD on catecholamine content and turnover. The histofluorometric method measures catecholamine fluorescence in tissues after exposure to formaldehyde vapours (Falck–Hillarp technique). This technique cannot distinguish between the fluorescent products of NE and α-MeNE.

The present HPLC—ED method has been applied to measuring the catecholamine content of the noradrenergic cell groups of the rabbit brain following α-MD administration. Distribution of NE, in general, was found to correlate well with the number of NE-containing cell bodies. However, in two regions, the A2 and A7 cell groups, some deviation was seen because these groups are also rich in NE-containing nerve terminals and dendrites. After α-MD administration, NE content measured in the A1 cell group was greatly reduced, but total catecholamine content was increased. This increase is predominantly due to a large accumulation of the metabolite α-MeNE. Whether the effects of α-MD reflect different turnover rates for NE in the noradrenergic cell groups is under investigation.

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