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DETERMINATION OF DOPA, DOPAMINE, DOPAC, EPINEPHRINE, NOREPINEPHRINE, α-MONOFLUOROMETHYLDOPA AND α-DIFLUOROMETHYLDOPA IN VARIOUS TISSUES OF MICE AND RATS USING REVERSED-PHASE ION-PAIR LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION*

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SUMMARY

A method for the determination of catecholic amino acids and amines by reversed-phase ion-pair high-performance liquid chromatography with electrochemical detection has been developed. By using octanesulfonic acid for ion pairing and by optimising ionic strength, pH and methanol concentration of the mobile phase, separation was achieved of 3,4-dihydroxyphenylalanine (DOPA), 3,4-dihydroxyphenylacetic acid (DOPAC), norepinephrine (NE), epinephrine (EPI), and dopamine (DA). α-Difluoromethyldopa (DFMD) and α-monofluoromethyldopa (MFMD), two potent enzyme-activated irreversible inhibitors of aromatic amino acid decarboxylase were also separated from the natural catechols. Concentrations of catechols and inhibitors were measured in brains, hearts and kidneys of mice treated with small repeated doses of MFMD. The method has also been applied to the determination of catechols in other organs such as prostates and seminal vesicles of rats and in smaller tissues like mesenteric arteries. A semi-automated procedure making use of an automatic sample processor and a digital integrator permitted the analysis of as many as sixty samples per day.

INTRODUCTION

Recently it has been shown that α-difluoromethyldopa (DFMD) and monofluoromethyldopa (MFMD) are potent enzyme-activated inhibitors of aromatic L-α-amino acid decarboxylase (AADC; E.C. 4.1.1.26) in vitro and in vivo [1—3]. Biochemical studies of these inhibitors called for a sensitive and simple method for the determination of the inhibitors and of 3,4-dihydroxyphenylalanine (DOPA), 3,4-dihydroxyphenylacetic acid (DOPAC), norepinephrine (NE), epinephrine (EPI) and dopamine (DA). A great variety of methods exist for the determination of natural catechols in tissues and body fluids. The widely used fluorometric method [4] lacks specificity while the radioenzy-

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matic methods [5, 6], although very sensitive, are not suited for the determination of the inhibitors, DFMD and MFMD. Gas—liquid chromatography [7, 8] allows the study of a great number of metabolites but requires derivatization before analysis. Liquid chromatography with pre-column derivatization with fluorescamine [9], *o*-phthaldialdehyde [10] or dansyl chloride [11], coupled with fluorometric detection has been employed for the analysis of catecholamines. Separation on a cation-exchange column followed by reaction with *o*-phthaldialdehyde has been used for the analysis of catechols in biological samples [12]. Derivatization, besides being time consuming in the case of the pre-column reactions, cannot be readily applied to compounds such as DFMD and MFMD in which the nucleophilicity of the carboxylic acid and the nitrogen groups has been greatly reduced. Therefore, high-performance liquid chromatography (HPLC) with electrochemical detection [13—15] which combines sensitivity with efficiency seemed the method of choice to measure concentrations of the inhibitors (DFMD and MFMD) and of the catechols in the same sample.

Reversed-phase ion-pair chromatography or soap chromatography [16] combines the advantages of reversed-phase and ion-exchange chromatography. By using octanesulfonic acid (OSA) as anionic modifier and by varying methanol concentration, ionic strength and pH of the mobile phase and the concentration of the anionic modifier, we established the optimal conditions for the separation of the catechols and of the inhibitors MFMD and DFMD. The consequences of inhibition of AADC by MFMD, the more potent inhibitor of AADC, on catechol levels in brain, heart and kidney of mice are reported In addition, catechol levels in prostates, seminal vesicles and mesenteric arteries of untreated rats have been measured.

MATERIALS AND METHODS

Chemicals

The catechol standards norepinephrine·HCl (NE), epinephrine (EPI) , dopamine·HCl (DA), 3,4-dihydroxyphenylalanine (DOPA), 3,4-dihydroxyphenylacetic acid (DOPAC), 3,4-dihydroxyphenylglycol (DOPEG), and α-methyldopa (α-MeDOPA) were purchased from Sigma (St. Louis, Mo., U.S.A.) and 3,4-dihydroxybenzylamine·HBr from Aldrich (Beerse, Belgium). D,L-α-Difluoromethyldopa (DFMD; RMI 71801) and D,L-α-monofluoromethyldopa (MFMD; RMI 71963) were synthesized in our Centre. Heptanesulfonic acid and octanesulfonic acid sodium salt were obtained from Eastman Kodak (Rochester, N.Y., U.S.A.) and ethylenediaminetetraacetic acid disodium salt (Na_2EDTA) from Carl Roth (Karlsruhe, G.F.R.). All other reagents were reagent grade from E. Merck (Darmstadt, G.F.R.).

Animals

Male CD1 albino mice (20—25 g) and male Sprague Dawley rats (200—300 g) from Charles River (Saint Aubin les Elbeuf, France) were used throughout these studies.

Chromatography

The high-performance liquid chromatograph consisted of a model 6000A

solvent delivery system, a U6K injector for the early experiments and a μBondapak C_{18} column (10-μm particle size range, 30 cm × 3.9 mm I.D.) all from Waters Assoc. (Milford, Mass., U.S.A.). For some experiments a LiChrosorb RP-18 column (10-μm particle size, 25 cm × 4.6 mm I.D.) from H. Knauer (Oberursel, G.F.R.) was used. An automatic sample injection system WISP model 710 from Waters Assoc. was used for the majority of the measurements. The pre-column (7 cm × 2 mm I.D.) filled with Partisil-10 ODS from Whatman (Clifton, N.J., U.S.A.) was refilled every week and was used to protect the main column. The detector was a Model LC-15 electrochemical detector from Bioanalytical Systems (West Lafayette, Ind., U.S.A.). The electrode was a wax-impregnated carbon paste. Its potential was maintained at + 0.9 V versus a silver—silver chloride reference electrode. The signal was recorded on a Kontron W+W recorder (Zürich, Switzerland) and the peak areas were determined with an Autolab System I integrator from Spectra Physics (Santa Clara, Calif., U.S.A.). The flow-rate was usually 1 ml/min.

Solvents

Water with resistivity greater than 10 MΩ cm^{-1} was obtained from a Millipore "Milli Q" system. Methanol, spectroscopic grade, was from E. Merck. The buffer consisted of 2 volumes of 0.02 *M* citric acid and 1 volume of 0.02 *M* Na_2HPO_4. The mobile phase was a 85:15 (v/v) mixture of this buffer with methanol (the amount of methanol depending on the capacity of the column) and made 2.5×10^{-3} *M* with sodium octylsulfonate and 5×10^{-5} *M* with Na_2EDTA. The pH was then adjusted to the desired value with concentrated phosphoric acid, usually to 3.25—3.35. The solvent mixture was filtered under vacuum through a 0.45-μm Millipore HA type filter before use.

Standards

Catechol stock solutions were prepared in 5×10^{-2} *M* $HClO_4$ containing 0.1% (w/v) $Na_2S_2O_5$ and 0.05% (w/v) Na_2EDTA at a concentration of 1 mg catechol per 100 ml. They were freshly prepared every 10 days and stored at 5°. Adequate standard solutions were obtained by diluting these stock solutions 50 times with 0.05 *N* $HClO_4$. Volumes of 25 μl or 50 μl were injected either with a Hamilton microsyringe or with the automatic sample injector.

Sample preparation

Extraction of the catechols followed a known procedure [17]. The tissues were homogenized in 2 or 4 ml (depending on the size of the tissues) of 0.4 *M* $HClO_4$ containing 0.05% (w/v) Na_2EDTA and 0.1% (w/v) $Na_2S_2O_5$, and α-MeDOPA (between 100 and 250 ng/ml depending on the sizes of the samples) as the internal standard. After centrifugation, the supernatant was added to 100 mg or 300 mg alumina and buffered to pH 8.0—8.4 with 0.1 *M* tricine containing 2.5% (w/v) Na_2EDTA and 2.1% (w/v) sodium hydroxide. The vials were shaken for 10 min on a reciprocal shaker, the supernatant removed and the alumina washed three times with 10 ml of water. Finally the catechols were eluted from the alumina by agitation for 10 min with 1 ml of 0.05 *M* $HClO_4$ containing 0.1% (w/v) $Na_2S_2O_5$ and 0.025% (w/v) Na_2EDTA. The supernatant was filtered off and either stored at —30° for later determination or transferred into minivials (250-μl size) of the automatic injector.

Recovery of the extraction procedure

Two series of experiments were conducted in order to determine the recovery of the various catechols from tissues. Known amounts of the catechols were dissolved in the 0.4 *M* $HClO_4$ solution and these solutions were used for tissue homogenization. In the first experiment, 2 ml of the solution were added to 100 mg of alumina and taken through the whole adsorption and extraction procedure outlined in the previous section. In a second experiment, 2 ml of the same solution were added to brains or hearts of mice, homogenized and extracted via the same procedure. The levels of catechols thus found were compared to the levels obtained in control brains or hearts extracted with perchloric acid. The recoveries from tissues were calculated from the difference between the values, with and without added standards.

RESULTS AND DISCUSSION

Determination of the chromatographic conditions

The goal of this study was to achieve after alumina adsorption the separation of all the catechols expected to be present in tissues. Apart from the inhibitors, DOPA, DOPAC, NE, EPI, DA and DOPEG are the most abundant. In order to make the method quantitative and reproducible, an internal standard, carried through the extraction procedure, was included. In similar studies α-MeDOPA and 3,4-dihydroxybenzylamine [18, 19] have been used. We chose α-MeDOPA because of its structural analogy with the inhibitors.

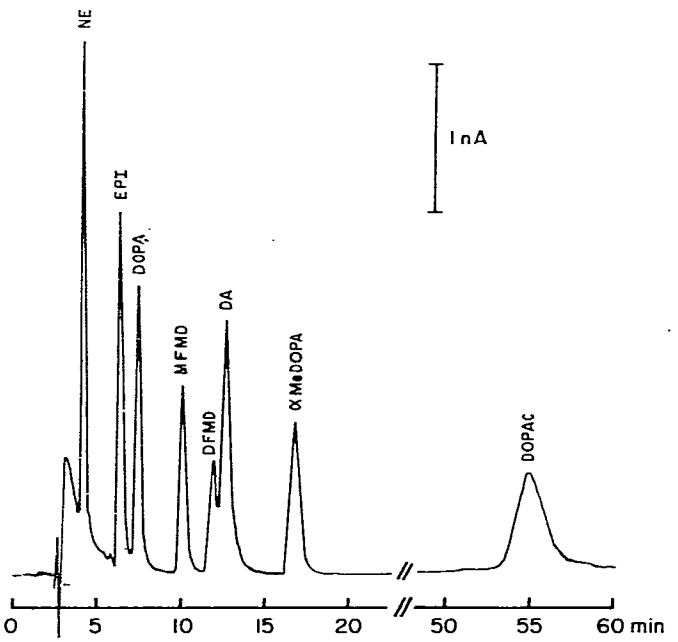

Fig. 1. Chromatogram of a standard mixture of 5 ng of each catechol. Column, LiChrosorb RP-18, 10 μm; mobile phase, 2:1 (v/v) mixture of citric acid (0.05 *M*) and Na_2HPO_4 (0.05 *M*) with EDTA (5×10^{-5} *M*); pH = 3.24; sample volume, 25 μl; flow-rate, 1 ml/min; temperature, ambient; electrode potential, + 0.9 V vs. Ag/AgCl reference electrode.

As was expected, the separation of catecholic compounds on reversed-phase packed columns depended strongly on the methanol composition, the ionic strength and the pH of the mobile phase. Fig. 1 shows the chromatogram obtained with a 2:1 (v/v) mixture of 0.05 *M* citric acid and 0.05 *M* Na_2HPO_4 (pH 3.24). These conditions are similar to those already published [18] for the separation of NE and DA. A clean separation of all the catechols of interest, with the exception of DFMD and DA, was obtained. This method has two major drawbacks: the excessive retention times and the elution of NE close to the elution front. It had been shown previously that by adding an anionic detergent [16] the separation pattern of the catechols can be drastically changed [20, 21]. Fig. 2 shows the chromatogram obtained by using the same buffer at lower ionic strength but after addition of heptanesulfonic acid (3.5×10^{-3} *M*) and 14% methanol. The desired separation of all the compounds has now been achieved, but the retention times especially for DA are still unsatisfactory. By using OSA at a lower concentration (2.5×10^{-3} *M*) fairly similar results are obtained, as is shown in Fig. 3. 3,4-Dihydroxyphenylglycol (DOPEG), which does not appear on this chromatogram, is eluted very close to the elution front and does not interfere with the other catechols of interest. In order to obtain the required separation, the ionic strength of the buffer, the concentration of the anionic detergent and especially the pH, were optimised. Fig. 4 shows the variation of the retention times of the cate-

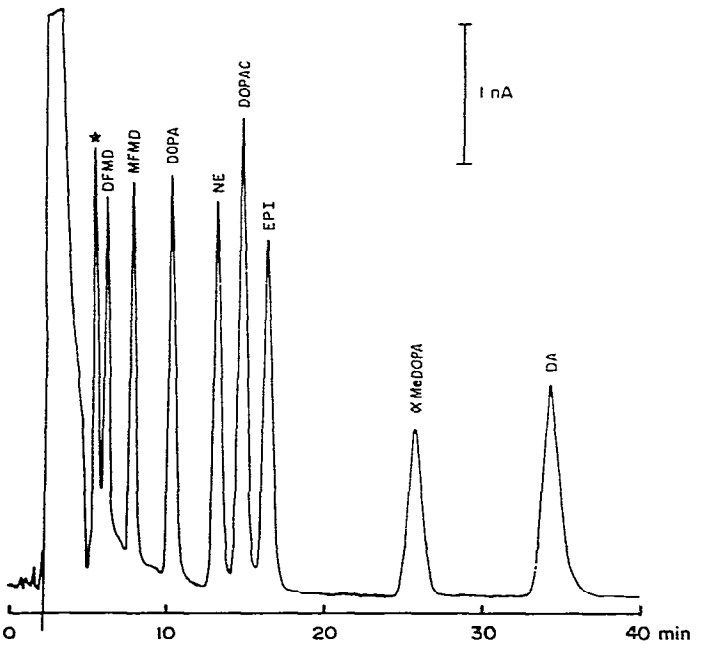

Fig. 2. Chromatogram of the same standard solution as in Fig. 1. Column LiChrosorb RP-18, 10 μm; mobile phase, 86:14 (v/v) mixture of the citrate—phosphate buffer (0.02 *M*) and methanol with heptanesulfonic acid (3.5×10^{-3} *M*) and EDTA (5×10^{-5} *M*); pH = 3.35; otherwise the conditions are the same as described in the legend to Fig. 1; ★, unknown peak deriving from the eluent.

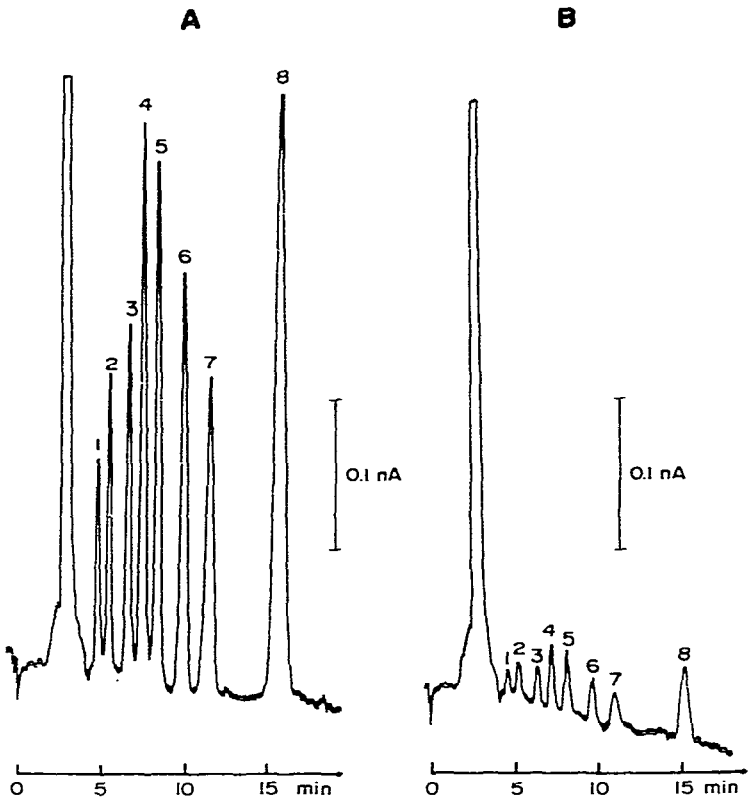

Fig. 3. Typical chromatograms showing the sensitivity of the method for standard solutions of catechols. Peaks: 1, DFMD; 2, MFMD; 3, DOPA; 4, DOPAC; 5, NE; 6, EPI; 7, α-MeDOPA 8, DA. Chromatographic conditions: column, μBondapak C_{18}; mobile phase, 87:13 (v/v) mixture of the citrate—phosphate buffer (0.02 *M*) and methanol with octanesulfonic acid (2.5×10^{-3} *M*) and EDTA (5×10^{-5} *M*); pH = 3.22; flow-rate, 1 ml/min; electrode potential, + 0.9 V vs. Ag/AgCl reference electrode; temperature, ambient. Trace A corresponds to 0.3 ng of 1, 2, 3 and 4; 0.35 ng of 5 and 6; 0.53 ng of 7 and 8. Trace B corresponds to 30 pg of 1, 2, 3 and 4; 35 pg of 5 and 6; 53 pg of 7 and 8.

chols with the pH of the eluent, when ionic strength, concentration of OSA and percentage of methanol are held constant. Two general statements can be made. Firstly, the retention times of the amines are independent of the pH of the eluent. This can be predicted from the fact that in the pH range of 2.4—3.5 the amino groups are completely protonated. Secondly, the retention times of the amino acids, especially those of DOPA and α-MeDOPA strongly increase as the pH decreases. This can be explained by the fact that ion-pair formation increases as the protonation of the carboxylic group increases (i.e. as the pH decreases) [22]. The pK_a values for DOPA (2.30 and 8.70 for the COOH and NH_2 groups respectively) [23], α-MeDOPA (2.4 and 8.8), DFMD (<1.7 and 7.05) and MFMD (1.7 and 8.10) [24] are in good agreement with the observed retention times. For MFMD and DFMD, the carboxylic groups remain mostly in the COO^- form and thus diminish the "amount" of ion-pair formation by repulsion with the negatively charged anionic detergent.

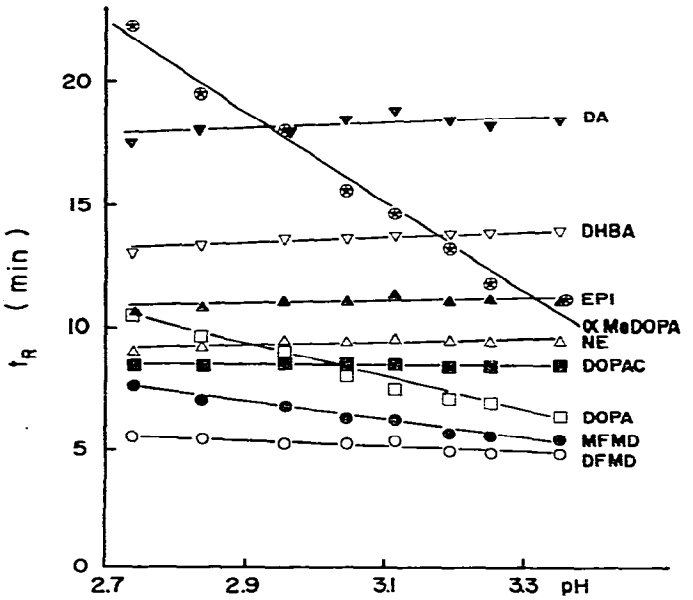

Fig. 4. Relationship between the retention times t_R and the pH of the mobile phase. Column, μBondapak C_{18}; mobile phase, 85:15 (v/v) mixture of the citrate—phosphate buffer (0.02 *M*) and methanol, with octanesulfonic acid (2.3×10^{-3} *M*); pH adjusted to the different values with phosphoric acid or sodium hydroxide; flow-rate, 1 ml/min; temperature: ambient; electrode potential: + 0.9 V vs. Ag/AgCl reference electrode.

These results show that different chromatographic conditions can lead to similar separations. Different anionic compounds, detergents or simple anions [25, 26] will give similar results with a different order of elution for the different catechols. A further consequence is that one can compensate for the loss of resolution of a column by decreasing the methanol concentration, by increasing the amount of the OSA or by changing both and adjusting the pH. In this case peak broadening occurs.

All chromatograms shown above were obtained after addition to the eluent of a small amount of Na_2EDTA (5×10^{-5} *M*) which strongly complexes metal ions, significantly improves the stability of the baseline and prevents the occurrence of spurious peaks due to contamination by metal ions [27—29]. Fig. 3 shows typical chromatograms obtained in the conditions outlined above with 300—500 pg in A and 30—50 pg in B of each catechol in a standard mixture. This sensitivity has been obtained by controlling carefully the eluent (addition of Na_2EDTA) and by applying to the wax-impregnated carbon paste electrode a working potential of + 0.9 V versus a Ag/AgCl reference electrode.

The routine assays were conducted under operating conditions (eluent composition) similar to those of Fig. 3, but at a lower sensitivity similar to that of Figs. 1 and 2. For most of the samples, an automatic sample injector and a digital integrator were used. On a 24 h per day working basis, as many as sixty samples could be assayed. The main problems were rapid loss of resolution for some columns and variation of the sensitivity of the carbon paste electrode.

However, as mentioned above, the loss of resolution can be partly overcome by adjusting the chromatographic parameters. Into some columns as many as 5000 samples could be injected without sizable loss of capacity or resolution. The carbon paste electrode can be easily repacked and reconditioned overnight.

For several reasons, especially changes of temperature and eluent composition, variations in sensitivity are observed. Therefore calibration has to be repeated at regular time intervals. The use of internal standards is necessary.

Calibration and recovery experiments

Various volumes of freshly prepared standard solutions were injected. Peak height measurements and peak areas calculated by the integrator were found to be linear for all the constituents in the range of 0.2 to 20 ng.

Several recovery experiments with 500-ng amounts of the catechols with and without tissues were conducted as described in the methods section. The results for brains are presented in Table I. Other experiments with 100 and 250 ng of each catechol gave similar results. The recoveries expressed as the ratio of recovery of α-MeDOPA and of the other catechols, in presence and in absence of tissues, remained constant with percentage variations ranging from 1.1 for DA to 6.6 for DOPAC as calculated from the differences of the ratios of Table I. The reproducibility expressed as the relative standard deviations (coefficient of variation) of the recoveries in absence of tissues varied from 3.5% for DOPA to 8.7% for DOPAC. As expected the acids such as DOPAC and the amino acids gave lower recoveries than the corresponding amines. These results are comparable to published values [26, 29—31]. α-MeDOPA appeared well-suited as an internal standard. Similar recovery experiments with hearts showed analogous results but with somewhat greater variations in the recovery, presumably due to the fact that the hearts were not as completely homogenized as the brains.

TABLE I

RECOVERY OF CATECHOLS FROM ALUMINA IN ABSENCE AND PRESENCE OF BRAIN TISSUE

	In absence of tissues*		In presence of tissues**	
	Recovery (%) ± S.D., $n = 5$	Mean ratio to α-MeDOPA	Recovery (%) ± S.D., $n = 10$	Mean ratio to α-MeDOPA
MFMD	57.5 ± 2.7	1.18	49.1 ± 3.1	1.21
DOPA	62.8 ± 2.2	1.08	51.6 ± 3.6	1.15
DOPAC	47.6 ± 4.1	1.42	39.2 ± 5.1	1.52
NE	77.9 ± 6.8	0.87	65.1 ± 7.3	0.91
EPI	76.7 ± 3.6	0.88	66.1 ± 3.5	0.90
α-MeDOPA	67.8 ± 5.7	1	59.5 ± 5.0	1
DA	74.2 ± 5.3	0.91	64.3 ± 9	0.92

*Values are the means of five determinations with 500 ng of each catechol.
**Values are the means of ten determinations with 500 ng of each catechol added to whole brain of mice and with ten control brains extracted as outlined in the text.

Calculation of the results

For most of the experiments, 100 ng (for mesenteric artery), 250 ng (for half brain and heart), 500 ng (for whole brain, heart, adrenal, kidney and prostate) of α-MeDOPA were added to the perchloric acid used for the homogenization of the tissues. To take into account the possible variation in sensitivity, one or two injections of the standard (same volumes were used for the unknown samples) were made after every sixth sample. From their peak heights or peak areas, the response factors were calculated for each constituent and updated after every sixth sample. With these response factors, the concentrations of the unknown catechols were calculated, then divided by the recovery of α-MeDOPA and multiplied with the ratio of recovery given in Table I to obtain the final amounts of catechols in ng or in ng per g of tissue weight. With this procedure, changes in sensitivity and variations in recovery from one sample to another were taken into account.

Applications of the method to different tissues

Catechols in brain, heart and kidney of mice after treatment with MFMD. Fig. 5 shows the chromatograms of brains of mice injected every 12 h for 3 days with 5 mg MFMD/kg (B) or with 25 mg/kg (C) by the intraperitoneal (i.p.) route. MFMD is not detectable in chromatogram B, but the DOPA peak is increased and the NE and DA peaks are decreased in comparison to the control. In chromatogram C, MFMD is clearly detectable and DOPA is markedly increased whereas the concentrations of DOPAC, NE and DA are

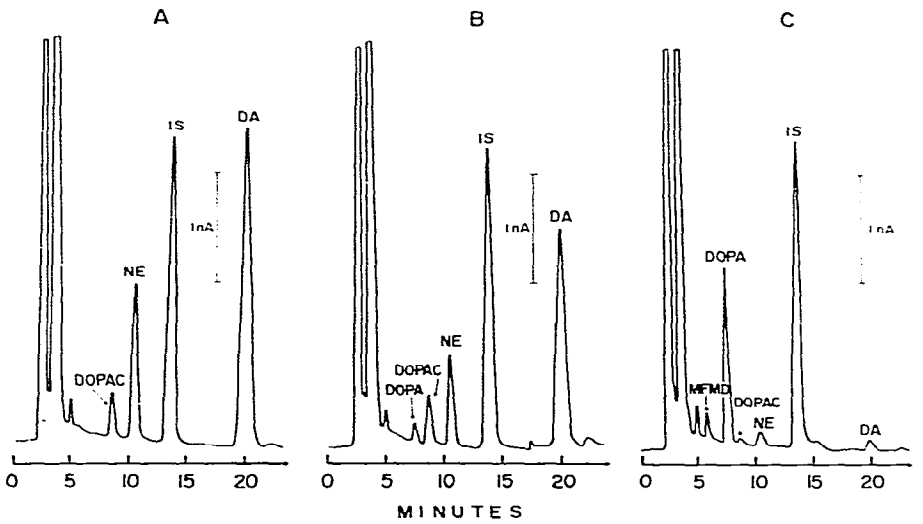

Fig. 5. Chromatograms of catechols from brains of mice, injected every 12 h for 3 days with saline (A), with 5 mg MFMD/kg/injection (B) or with 25 mg MFMD/kg/injection (C). α-MeDOPA as internal standard (IS). Chromatographic conditions: column, μBondapak $C_{1\,8}$; mobile phase, 87:13 (v/v) mixture of the citrate—phosphate buffer (0.02 *M*) and methanol with octanesulfonic acid (2.5×10^{-3} *M*) and EDTA (5×10^{-5} *M*); pH = 3.25; flow-rate, 1 ml/min; electrode potential, + 0.9 V vs. Ag/AgCl reference electrode; temperature, ambient.

considerably reduced. Fig. 6 shows the effect of four different doses of MFMD on MFMD, DOPA, DOPAC, NE and DA. The results are expressed in ng/g wet tissue ± S.E.M. after correction for recovery. The results clearly indicate the potency of MFMD as an AADC inhibitor in mouse brain. The levels of NE and DA found in the control animals are in good agreement with published values [32].

Similar effects were obtained for heart, kidney and adrenals. The catechol levels found in heart and kidney at the two highest doses (6 × 5 and 6 × 25 mg/kg) are presented in Table II. At these doses, MFMD can be easily determined, the concentrations of NE and, to a lesser extent, DA decrease and there is a significant increase in DOPA.

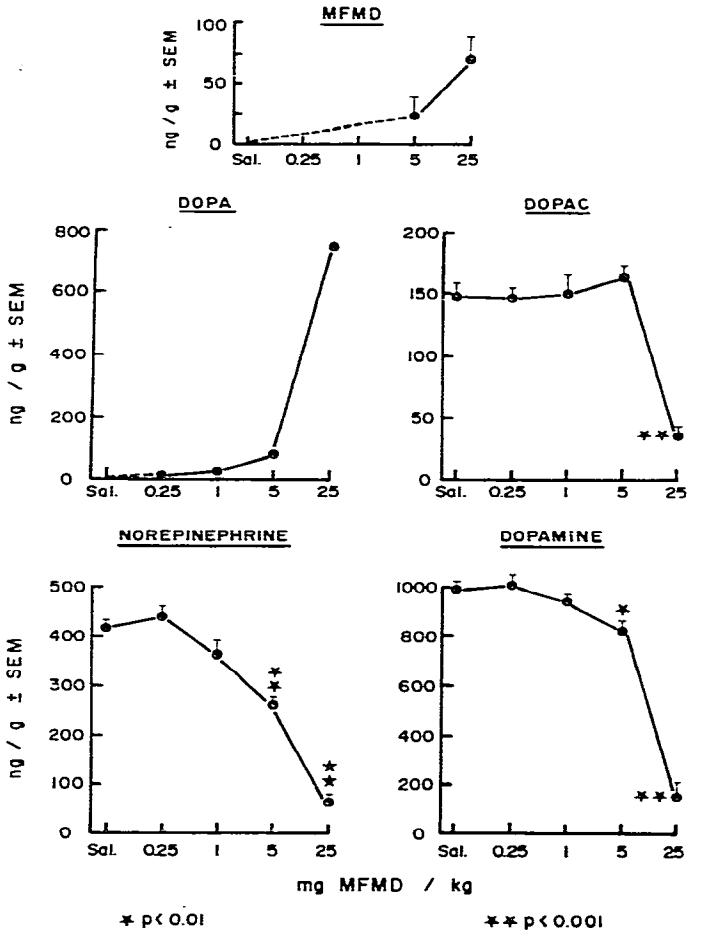

Fig. 6. Effect of repeated administration of small doses of MFMD on DOPA, DOPAC, NE, DA and MFMD content of mouse brain. Groups of 5 mice were injected i.p. every 12 h for 3 days with increasing doses of MFMD. They were sacrificed 6 h after the last injection. Five mice injected with saline served as controls. Half brains were homogenized in 0.4 *M* $HClO_4$ and processed as described in the experimental procedure. Values are expressed in ng/g wet weight ± S.E.M. * $p < 0.01$ and **, $p < 0.001$, Student's *t*-test.

TABLE II

CATECHOL LEVELS IN HEART AND KIDNEY OF MICE AFTER TREATMENT WITH MFMD

Values are given in ng per g wet weight ± S.E.M. (n = 5). Statistically significant differences compared to controls as determined by Student's t-test are shown by (*) $p < 0.05$, (**) $p < 0.005$, (***) $p < 0.001$, NS = not significant. Mice were injected every 12 h for 3 days via the i.p. route. Control animals received an equivalent volume of distilled water. Animals were sacrificed 6 h after the last dose.

	MFMD	DOPA	NE	EPI	DA
Heart					
Control	—	32 ± 14	650 ± 48	—	30 ± 6
6 × 5 mg/kg	30 ± 3	115 ± 21 (*)	400 ± 23 (**)	—	⩽15 ± 5 (*)
6 × 25 mg/kg	142 ± 40	248 ± 23 (***)	103 ± 23 (***)	—	⩽20 ± 10 (NS)
Kidney					
Control	—	⩽2.5 ± 2.5	300 ± 22	20 ± 17	13.3 ± 4.9
6 × 5 mg/kg	21 ± 5	181 ± 27 (***)	201 ± 32 (*)	61 ± 40 (NS)	11.5 ± 4.0 (NS)
6 × 25 mg/kg	193 ± 66	207 ± 23 (***)	70 ± 14 (***)	11 ± 6.5 (NS)	4.9 ± 0.5 (*)

Catechol determination in ventral prostate of rats. For the determination of catechols in the ventral prostate of male adult rats, two different methods were used: Method A followed essentially the experimental procedure outlined previously in the text by using 2 ml of 0.4 M $HClO_4$ (with $Na_2S_2O_5$, EDTA and 500 ng of α-MeDOPA as internal standard) for the tissue homogenization, then adsorption on 100 mg of alumina and finally extraction with 1 ml 0.05 M $HClO_4$. Method B used 2 ml of 5% trichloroacetic acid (with $Na_2S_2O_5$, EDTA and α-MeDOPA) for tissue homogenization. Table III presents the results expressed in ng/g tissue ± S.E.M. The mean tissue weight was 591 ± 25 mg (n = 9) in method A and 556 ± 25 mg (n = 16) in method B. The values found for NE are in good agreement with published values [33] obtained with a fluorometric method, but the values found for DA are much lower than the values reported. The HPLC method of catechol determination in prostate allowed us to study the effect on catechol levels of a combined treatment with DFMD and DOPA as compared to treatment with DOPA and DFMD alone [34].

Catechol concentrations in seminal vesicles. Seminal vesicles of rats were homogenized using the same methods A and B as were used for the prostates. The mean tissue weight after removing secretions was 169 ± 9 mg (n = 9) in method A and 167 ± 7 (n = 10) in method B. The results presented in Table IV agree well with published values for NE [35]. There are no significant differences between the values found by the two methods.

TABLE III

CATECHOL LEVELS IN RAT VENTRAL PROSTATE AND SEMINAL VESICLE

	DOPA		DOPAC		NE		DA	
	Method A*	Method B**	Method A	Method B	Method A	Method B	Method A	Method B
Prostate*** ng/g wet weight ± S.E.M.	≤10 ± 5	≤10 ± 5	<5	<5	666 ± 56	637 ± 20	43 ± 6	30 ± 3
Seminal vesicle† ng/g wet weight ± SEM	<5	<5	5 ± 5	19 ± 5	1752 ± 90	1795 ± 76	167 ± 13	152 ± 11

*Tissues were homogenized in 2 ml of 0.4 *M* $HClO_4$ with 500 ng of α-MeDOPA as internal standard. After adsorption on alumina the catechols were extracted with 1 ml of 0.05 *M* $HClO_4$ and processed as described in the text.

**Tissues were homogenized in 5% (w/v) trichloroacetic acid and then processed as described in the first footnote.

***Values are the means of $n = 9$ prostates for method A and $n = 16$ for method B.

†Values are the means of $n = 9$ seminal vesicles for method A and $n = 10$ for method B.

Determination of catechols in small tissue samples: application of the method to mesenteric arteries. For small tissue samples, such as the mesenteric arteries, portal veins and brain areas the procedure outlined in the experimental part has been scaled down. 0.5 ml of 0.4 *M* $HClO_4$ containing 100 ng of α-MeDOPA as internal standard (with $Na_2S_2O_5$ and EDTA) were used for the tissue homogenization (for about 40 mg of tissue). Adsorption was done on 25 mg of alumina and back extraction with 100 μl of 0.1 *M* $HClO_4$. Aliquots (50 μl) of this extract were injected and the ionic strength of the buffer used for the preparation of the eluent was increased to 0.035 *M* in order to avoid changes in baseline and retention times. Table IV shows the results obtained with mesenteric arteries. Their mean weight was 40 ± 4 mg ($n = 9$). This allowed us to study the effect of MFMD treatment on the catecholamine levels in mesenteric arteries and in portal veins [36].

General remarks. From the above examples, it is apparent that the homogenization and extraction procedure has to be adapted to the tissue size of each organ. An internal standard is essential for verification of the reproducibility of the extraction procedure within an experiment and also allows the

TABLE IV

CATECHOL LEVELS IN MESENTERIC ARTERIES

	DOPA	DOPAC	NE	DA
ng per organ ± S.E.M. ($n = 9$)	7.6 ± 3	10.4 ± 1.3	58.3 ± 4.6	6.4 ± 1.2
ng per g wet weight ± S.E.M. ($n = 9$)	183 ± 66	279 ± 42	1600 ± 240	169 ± 97

comparison of values from experiments performed at different times. For example, for mouse brain, the NE and DA values found over a five month period remained constant, 406 ± 6 and 1012 ± 16 ng/g, respectively (mean ± S.E.M., $n = 74$).

By scaling down the volumes used for the homogenization and extraction procedure, the catechol concentrations in small tissue samples can be easily measured. By working at a higher sensitivity such as in chromatogram B of Fig. 3 and by carefully controlling the temperature, 50—100 pg of catechols may be measured with a signal-to-noise ratio greater than 5. This sensitivity is adequate for the measurements of catechols in biological fluids, such as serum or plasma [26, 30, 31].

By using an automatic injector and a digital peak integrator fifty to sixty samples could be analyzed per day.

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REFERENCES

1 M.G. Palfreyman, C. Danzin, P. Bey, M.J. Jung, G. Ribereau-Gayon, M. Aubry, J.P. Vevert and A. Sjoerdsma, J. Neurochem., 31 (1978) 927.
2 J. Kollonitsch, A.A. Patchett, S. Marburg, A.L. Maycock, L.M. Perkins, G.A. Doldouras, D.E. Duggan and S.D. Aster, Nature (London), 274 (1978) 906.
3 M.J. Jung, M.G. Palfreyman, J. Wagner, P. Bey, G. Ribereau-Gayon, M. Zraika and J. Koch-Weser, Life Sci., 24 (1979) 1037.
4 A.H. Anton and D.F. Sayre, J. Pharmacol. Exp. Ther., 138 (1962) 360.
5 A.C. Cuello, R. Hiley and L.L. Iversen, J. Neurochem., 21 (1973) 1337.
6 V. Werse and J. Kopin, Life Sci., 19 (1976) 673.
7 D.B. Calne, F. Karoum, C.R.J. Ruthven and M. Sandler, Brit. J. Pharmacol., 37 (1969) 57.
8 J.C. Lhuguenot and B.F. Maume, J. Chromatogr. Sci., 12 (1974) 411.
9 K. Imai and Z. Tamura, Clin. Chim. Acta, 85 (1978) 1.
10 T.P. Davis, C.W. Gehrke, C.W. Gehrke, Jr., T.D. Cunningham, K.C. Kuo, K.O. Gerhardt, H.D. Johnson and C.H. Williams, Clin. Chem., 24 (1978) 1317.
11 G. Schwedt and H.H. Bussemas, Z. Anal. Chem., 283 (1977) 23.
12 P.M. Froehlich and T.D. Cunningham, Anal. Chim. Acta, 97 (1978) 357.
13 P.T. Kissinger, Anal. Chem., 49 (1977) 447A.
14 R.M. Riggin and P.T. Kissinger, Anal. Chem., 49 (1977) 2109.
15 R. Keller, A. Oke, I. Mefford and R.N. Adams, Life Sci., 19 (1976) 995.
16 J.H. Knox and J. Jurand, J. Chromatogr., 125 (1976) 89.
17 M.K. Schellenberger and J.H. Gordon, Anal. Biochem., 39 (1971) 356.
18 L.J. Felice, J.D. Felice and P.T. Kissinger, J. Neurochem., 31 (1978) 1461.
19 L.D. Mell, Jr. and A.B. Gustafson, Clin. Chem., 24 (1978) 1290.
20 J. Mitchell and C.J. Coscia, J. Chromatogr., 145 (1978) 295.
21 T.S. Moyer and N.S. Jiang, J. Chromatogr., 153 (1978) 365.
22 Cs. Horváth and W. Melander, J. Chromatogr. Sci., 15 (1977) 393.
23 J.E. Gorton and R.F. Jameson, J. Chem. Soc. A, (1968) 2615.
24 P. Bey and J. Wagner, unpublished results.
25 I. Molnar and Cs. Horváth, Clin. Chem., 22 (1976) 1497.

26 C. Freed and P.A. Asmus, J. Neurochem., 32 (1979) 163.
27 H. Hashimoto and Y. Maruyama, J. Chromatogr., 152 (1978) 387.
28 J.P. Crombeen, J.C. Kraak and H. Poppe, J. Chromatogr., 167 (1978) 219.
29 C. Refshauge, P.T. Kissinger, R. Dreiling, L. Blank, R. Freeman and R.N. Adams, Life Sci., 14 (1974) 311.
30 H. Hallman, L.O. Fanubo, B. Hamberger and G. Jonsson, Life Sci., 23 (1978) 1049.
31 C. Hanson, G. Agrup, H. Rorsman, A.M. Rosengren, E. Rosengren and L.E. Edholm, J. Chromatogr., 162 (1979) 7.
32 S. Sasa and C. L. Blank, Anal. Chem., 49 (1977) 354.
33 R.B. Rastogi, R.A. Agarwal, K.P. Bhargava and R.L. Singhal, Can. J. Physiol. Pharmacol., 55 (1977) 1015.
34 C. Danzin, M.G. Palfreyman and J. Wagner, in preparation.
35 A.R. Wakade, A.G. Garcia and S.M. Kirpekan, J. Pharmacol. Exp. Ther., 193 (1975) 424.
36 J.R. Fozard, M.G. Palfreyman, M.L. Part, M. Spedding, J. Wagner and J.K. Woodward, Brit. J. Pharmacol., (1979) in press.