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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF S-ADENOSYLMETHIONINE AND ITS METABOLITES IN RAT TISSUES: INTERRELATIONSHIP WITH CHANGES IN BIOGENIC CATECHOL LEVELS FOLLOWING TREATMENT WITH L-DOPA

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SUMMARY

A method is described for the simultaneous analysis of S-adenosylmethionine (SAM) and its metabolites, S-adenosylhomocysteine (SAH) and decarboxylated S-adenosylmethionine along with the natural polyamines, putrescine, spermidine and spermine. The separation is obtained by a reversed-phase ion-pair liquid chromatographic procedure with gradient elution followed by dual detection. The UV absorbance at 254 nm is used for the analysis of SAM and of the SAM metabolites, whereas the polyamines and some major amino acids, *e.g.*, methionine, tyrosine and tryptophan, are analyzed by fluorescence detection after UV-cell derivatization with *o*-phthalaldehyde. A separate ion-pair reversed-phase high-performance liquid chromatographic (HPLC) procedure using isocratic elution and electrochemical detection is employed to analyse in the same tissue extracts the catechols and 5-hydroxyindoles, 3,4-dihydroxyphenylalanine (DOPA), dopamine, norepinephrine, 3,4-dihydroxyphenylacetic acid, homovanillic acid, 4-hydroxy-3-methoxyphenylalanine, tryptophan, 5-hydroxytryptophan, serotonin and 5-hydroxyindolacetic acid. The sample preparation for the two HPLC procedures requires only homogenization of the tissues in perchloric acid and centrifugation before injection onto the column. The two chromatographic procedures have been applied to study the interrelationship, in various tissues of rats, between the SAM and SAH levels and the biogenic catechols after different treatments with L-DOPA alone or in combination with α-monofluoromethyl-DOPA, a potent enzyme-activated irreversible inhibitor of aromatic L-amino acid decarboxylase.

INTRODUCTION

L-DOPA (3,4-dihydroxyphenylalanine) is widely used, alone or in combination with peripherally selective inhibitors of aromatic L-α-amino acid decarboxylase (AADC; E.C. 4.1.1.26), for the treatment of Parkinson's disease[1]. In rats, such treatments lead to increased concentrations of dopamine (DA) in the brain[2], although the concentration of norepinephrine (NE) was found either to be unchanged[2,3] or in-

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creased[4]. One of the major metabolic pathways for the catabolism of DOPA and its metabolites is O-methylation to 3-O-methyl-DOPA (3-OMeDOPA) and the corresponding methylated products by the enzyme catechol-O-methyl transferase (COMT; E.C. 2.1.1.6). Furthermore, it has been shown[4,5] that the treatment of rats with L-DOPA leads to significant decreases in the levels of S-adenosylmethionine (SAM), the methyl donor in this biochemical process. This depletion of SAM is accompanied by a concomitant increase of its demethylated metabolite, S-adenosylhomocysteine (SAH)[6] and a decrease in methionine, its precursor[7]. Since S-adenosylmethionine is involved in several other biochemical processes, its variation may lead to various other biochemical effects[8,9].

Several procedures for the analysis of SAM and of its metabolites have been described. Most of them either need a complex and tedious sample preparation or do not allow the simultaneous determination of all the SAM metabolites[8,10–13]. The use of cation-exchange and reversed-phase packings has greatly improved the separation procedures[6,12–16]. In a previous paper[17], we have shown how reversed-phase ion-pair high-performance liquid chromatography (HPLC) can be used to advantage for the simultaneous analysis of all the major SAM metabolites. The described procedure uses gradient elution and UV detection at 254 nm followed by derivatization with *o*-phthalaldehyde (OPA) and subsequent fluorescence detection. Moreover, the method allows the simultaneous analysis in the same run of the natural polyamines and some major amino acids. We have shown that the separation can be optimized by carefully adjusting the various parameters which govern the retention mechanisms[18], *i.e.*, the nature and concentration of the anionic modifier, the ionic strength of the eluent, the temperature and especially the pH of the mobile phases.

Furthermore, in previous reports we have shown how the reversed-phase ion-pair chromatographic procedures using electrochemical detection could be tailored to obtain the desired separation of the catechol and 5-hydroxyindole derivatives involved in regulation of the central and peripheral nervous system[19,20].

The present work demonstrates that the above two chromatographic procedures can be greatly improved by the use of more efficient packings, namely 5-μm spherical particles. It also helps to understand better the interaction of SAM-related processes. To this end, the two methods have been applied to study, in various tissues of rats, the effects of treatment with L-DOPA alone and in combination with α-monofluoromethyl-DOPA (MFMD). MFMD has been shown to be a potent enzyme-activated irreversible inhibitor of AADC[21] which at low doses shows selectivity for the peripheral enzyme[22]. The gradient elution procedure with UV and fluorescence detection allows a measurement of the levels of SAM and its metabolites SAH, 5′-deoxy-5′-methylthioadenosine (MTA) and the decarboxylated analogue, dc-SAM, along with the polyamines, putrescine, spermidine and spermine. In the same run, the amino acids tyrosine, tryptophan and methionine can also be determined. Concurrently, HPLC analysis with electrochemical detection of the same samples gives the levels of the major catechols, DOPA, DA, NE, epinephrine (EPI), 3,4-dihydroxyphenylacetic acid (DOPAC) and their metabolites, 3-OMeDOPA, 4-hydroxy-3-methoxy-phenylacetic acid (HVA), together with the major 5-hydroxyindole precursors and derivatives, *i.e.*, tryptophan, 5-hydroxytryptophan (5-HTP), serotonin (5-HT) and 5-hydroxyindolacetic acid (5-HIAA). Both HPLC procedures use a simplified sample preparation by directly injecting into the column the clear tissue extracts obtained after homogenization in perchloric acid and centrifugation.

The results obtained permit new insights into the interrelationship of the various biochemical pathways involving SAM.

MATERIALS AND METHODS

Chemicals

S-Adenosyl-L-methionine chloride salt (SAM), S-adenosyl-L-homocysteine (SAH), 5′-deoxy-5′-methylthioadenosine (MTA), S-adenosyl-L-ethionine iodide salt (SAE), L-methionine (Met), DL-ethionine (Eth), arginine hydrochloride (Arg), lysine hydrochloride (Lys), homocarnosine hydrosulphate (HomoCarn), putrescine dihydrochloride (Put), spermidine trihydrochloride (Spd), spermine tetrahydrochloride (Spm) and 1,7-diaminoheptane dihydrochloride (Di Hept) were products of Sigma (St. Louis, MO, U.S.A.). The catechol and indole precursors and metabolites, *i.e.*, tyrosine (Tyr), L-DOPA, α-methyl-DOPA (α-MeDOPA), norepinephrine (NE), epinephrine (EPI), dopamine hydrochloride (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), 4-hydroxy-3-methoxyphenylacetic acid (HVA), 4-hydroxy-3-methoxyphenylalanine (3-OMeDOPA), tryptophan (Trp), serotonin hydrochloride (5-HT), 5-hydroxytryptophan (5-HTP) and 5-hydroxyindolacetic acid (5-HIAA) were all products from Sigma. S-(5′-Deoxy-5′-adenosyl)-3-methylthiopropylamine bisulphate (dc-SAM), S-(5′-deoxy-5′-adenosyl)-3-thiopropylamine bisulphate (dc-SAH) and S-(5′-deoxy-5′-adenosyl)-3-ethylthiopropylamine iodide (dc-SAE) were prepared following published procedures[23]. D,L-α-MFMD (MDL 71963) was synthesized in our Centre[24]. The N-acetylpolyamines, N-acetylputrescine (N-AcPut), N^1- and N^8-acetylspermidine (N^1-AcSpd, N^8-AcSpd) were provided by Dr. N. Seiler (MDRI, Strasbourg Center). Octanesulphonic acid sodium salt (OSA) was obtained from Eastman-Kodak (Rochester, NY, U.S.A.), whereas *o*-phthalaldehyde (OPA) and ethylenediaminetetraacetic acid disodium salt (EDTA) were from Carl Roth (Karlsruhe, F.R.G.).

Acetonitrile (LiChrosolv), methanol (Uvasol) and all the other chemicals were analytical grade from E. Merck (Darmstadt, F.R.G.).

Animals

Male Sprague–Dawley rats (200–300 g) from Charles River, France were used throughout these studies.

LC analysis of SAM analogues and polyamines

Apparatus. The HPLC system used was similar to the one previously described[17]. It consisted mainly of two Model 6000A pumps, a Model 660 solvent programmer, a Model 440 UV absorbance detector and a WISP 710A automatic sample injector, all from Waters (Milford, MA, U.S.A.). The set-up for the derivatization with OPA was identical to the one previously described[17]. The two signals of the UV detector and of the fluorescence detector were recorded on an Omniscribe recorder from Houston Instruments (Austin, TX, U.S.A.) and integrated with two SP4100 digital integrators from Spectra Physics (Santa Clara, CA, U.S.A.). The main difference from the already described system was the use of an Ultrasphere ion-pair column, (particle size 5 μm, 250 × 4.6 mm) from Beckman (Berkeley, CA, U.S.A.) protected with a guard column (70 × 2 mm) filled with Co:Pell ODS (particle size

30–38 μm) from Whatman (Clifton, NJ, U.S.A.). The flow-rate was 1.5 ml/min. The two columns were thermostatted at 40 ± 0.5°C in a jacket connected to a water-bath.

Mobile phase. As described[17], a gradient elution system was used. Mobile phase A was 0.1 M NaH_2PO_4–acetonitrile (98:2, v/v) and contained $8 \cdot 10^{-3}$ M OSA. The pH was adjusted to 2.65 with 10.5 ml of 3 M H_3PO_4. It was usually filtered under vacuum with a 0.45-μm Millipore HA filter before the addition of acetonitrile and after mixing with acetonitrile was carefully degassed on a ultrasonic bath. Mobile phase B was a mixture of 740 ml of 0.15 M NaH_2PO_4 and 260 ml of acetonitrile and contained OSA at $8 \cdot 10^{-3}$ M; its pH was adjusted to 3.25 with 10.5 ml of 3 M H_3PO_4. It was filtered under vacuum with a 0.5-μm Millipore FHUP filter and kept for 10 min in an ultrasonic bath before use. A linear gradient was used starting with 85% of eluent A and 15% of eluent B and leading in 30 min to the final conditions of 100% of eluent B. At this time, an automatic resetting to the initial conditions was triggered by the WISP, followed by a stabilization time of 10 min before the next injection was performed. The use of the automatic sample injector to initiate the solvent gradient led to an excellent reproducibility. Small amounts of EDTA, usually $1 \cdot 10^{-4}$ M, were added to eluent A in order to compensate for the slight drift of the baseline in the UV detection, resulting from differences in absorption between eluents A and B.

Acetonitrile was distilled over P_2O_5, and water, obtained from a Millipore "Milli Q" system, was redistilled over phosphoric acid in order to eliminate contaminations by volatile amines.

LC analysis of catechol and indole analogues

Apparatus. The HPLC system was similar to the one described[19,20] and differed only in the use of an Ultrasphere ion-pair column (particle size 5 μm, 250 × 4.6 mm) identical to the one described above. The detector was a Model LC-2A electrochemical detector from Bioanalytical systems (West-Lafayette, IN, U.S.A.) with a glassy carbon TL-5 electrode whose potential was maintained at 0.8–0.9 V *versus* a silver–silver chloride reference electrode. The signal, after reduction by a factor of 100 with a potentiometric divider, was recorded and integrated with a dual-channel Model HP-3388 from Hewlett-Packard (Palo Alto, CA, U.S.A.). The flow-rate was usually 1 ml/min and the temperature adjusted to 28–29°C.

Mobile phase. The eluent, prepared as described previously[20], comprised 0.1 M NaH_2PO_4–methanol (83.5:16.5, v/v) with $2.6 \cdot 10^{-3}$ M OSA, $1 \cdot 10^{-4}$ M EDTA and $2.5 \cdot 10^{-4}$ M triethylamine (Et_3N). The pH was carefully adjusted to 3.35 with 3 M H_3PO_4.

Standards

Stock solutions of the different SAM analogues and the polyamines were prepared by dissolving 0.5–3 mg of the different compounds in 50 ml of 0.05 M $HClO_4$ containing 0.05% (w/v) $Na_2S_2O_5$ and 0.01% (w/v) EDTA. Adequate standard solutions were obtained by diluting these stock solutions five times. Fresh diluted standards had to be prepared every 2–3 weeks because Trp, Tyr and some of the catechols and indoles added were prone to decomposition. Usually, aliquots of 15–20 μl of the standard solutions were injected.

The stock solutions of catechols and indoles were prepared as previously described[20] by dissolving 1–2 mg in 100 ml 0.1 *M* $HClO_4$ containing 0.1% (w/v) $Na_2S_2O_5$ and 0.01% (w/v) EDTA. Standard solutions were obtained by diluting these stock solutions 50 times in 0.1 *M* $HClO_4$ (with $Na_2S_2O_5$ and EDTA) and were freshly prepared every week. Aliquots of 1–5 μl of these standard solutions were injected.

Sample preparation

The same sample extracts were used for the SAM–polyamine analysis and for the catechol–indole determination. The different tissues of rats were homogenized in 0.4 *M* $HClO_4$ containing 0.15% (w/v) $Na_2S_2O_5$ and 0.05% (w/v) EDTA. Usually, the following volumes of $HClO_4$ were used for homogenization; 4 ml for brain ($\approx$2 g of tissue), heart (0.7–0.8 g), liver (1.5–2 g), lung (0.5–1 g), kidney (0.7–1 g) and adrenals ($\approx$0.05 g); 5 ml for pancreas (0.5–1 g) and ventral prostate (0.2–0.4 g). After centrifugation the clear supernatant was removed and stored at 0–5°C. Samples can be stored up to 1 week under these conditions without appreciable loss (< 10%) for the indole and 5-hydroxyindole derivatives. At −80°C, samples can be stored for up to 1 month. Typically, 10–50 μl of these acid extracts were directly injected for the SAM and polyamine analysis, and concurrently an equal or smaller volume onto the other HPLC system for the catechol and indole determination.

Calculations

As already stated[17], the responses of the UV signal and of the fluorescence signal were found to be linear over the range investigated. Since the recovery of the SAM analogues and polyamines was found to be quantitative[17], no internal standard or only occasionally an internal standard, 1,7-diaminoheptane, was included in the $HClO_4$ used for the homogenization. Usually, an external standard containing all the compounds of interest was injected every tenth sample and used for the calibration. The commercially available SAM and to a lesser extent SAE were impure, containing up to 30% MTA and SAH [or 5′-deoxy-5′-ethylthioadenosine (ETA) and SAH for SAE]. Since attempts at purification proved unsuccessful, we used the commercially available compounds but corrected the response factors of SAM, SAH and MTA by taking into account the concentrations of the contaminants that we determined by HPLC. The final tissue contents are expressed in nmol per g wet weight.

For the catechols and 5-hydroxyindoles, we used α-MeDOPA as an internal standard. In a previous paper[20] we described extensive studies of the recovery of the various compounds from the crude homogenates and the relative recoveries of the different compounds in comparison to that of the internal standard, α-MeDOPA. Therefore, an external standard together with the complete mixture was injected every eighth sample to compensate for the slight decrease in sensitivity which may be observed for the glassy carbon electrode. The actual amounts (in ng per g or nmol per g of tissue) of the different compounds were calculated by taking into account their relative recoveries in comparison to the internal standard.

Fig. 1. Chromatograms of the standard solution of SAM analogues and polyamines with UV detection at 254 nm and fluorescence detection after *o*-phthalaldehyde derivatization. For details of the chromatographic conditions see Materials and methods. Amounts in nmoles: UV detection, Tyr (1.5), 5-HIAA (0.6), HVA (1.4), 3-OMeDOPA (1.22), DA (0.71), SAH (0.13), SAM (0.12), SAE (0.11), MTA (0.11), dc-SAH (0.12), Trp (0.68), dc-SAM (0.08), dc-SAE (0.17); fluorescence detection, N-AcPut (0.31), Lys (0.47), Met (0.59), Tyr (1.5), 3-OMeDOPA (1.22), DA (0.71), Arg (0.68), HomoCarn (0.42), Eth (0.58), Put (0.37), N¹-AcSpd (0.35), N⁸-AcSpd (0.22), Trp (0.68), Di Hept (0.34), Spd (0.39), Spm (0.59). The sensitivity of the UV detector was set at 0.1 and the attenuation of the SP4100 integrator at 8 with the input selector at 100 mV. The sensitivity of the Aminco fluoromonitor was set at 100 with the input range set at 1 V and the attenuation of the SP4100 integrator at 16.

RESULTS AND DISCUSSION

Optimization of the chromatographic conditions

Simultaneous determination of SAM analogues and polyamines. In a previous paper[17] we showed that, by using a gradient elution and by carefully adjusting the

TABLE I

CHROMATOGRAPHIC PROPERTIES OF THE VARIOUS SAM ANALOGUES AND OF THE POLYAMINES AND AMINO ACIDS

Compound	*UV detection at 254 nm*		*Compound*	*Fluorescence detection*	
	Retention time (min)	*Detection limit**		*Retention time (min)*	*Detection limit**
DOPA**	5.1	50	N-AcPut	5.05	6
Tyr	8.1	50	DOPA**	5.45	25
5-HIAA	9.3	4	Lys	5.50	4
HVA	11.64	14	Met	7.25	10
3-OMeDOPA	11.8	80	Tyr	8.45	12
DA	14.02	32	3-OMeDOPA	12.1	12
SAH	15.24	0.8	DA	14.4	5
SAM	17.50	1.0	Arg	14.93	6
SAE***	18.16	0.8	HomoCarn	15.73	4
	18.32		Eth	16.11	7
MTA	19.78	0.8	Put	17.41	3.5
dc-SAH	20.60	0.8	N^1-AcSpd	20.21	3.5
Trp	21.75	4	N^8-AcSpd	20.80	3.0
dc-SAM§	23.64	0.9	Trp	22.11	9
ETA	23.90	–	Di Hept	22.90	4.5
dc-SAE§	24.21	1.2	Spd	24.88	4
			Spm	27.76	5.5

* Expressed in pmol by considering a signal-to-noise ratio of 2, for the chromatograms given in Fig. 1. The response factors of SAH, SAM, MTA and SAE were corrected by taking into account the purity of SAM and SAE as obtained by separate analysis.

** The retention time of DOPA was obtained from a separate run and was not included in this standard because of its interference with lysine.

*** Two peaks of equal heights are obtained (see discussion in ref. 40).

§ dc-SAM and dc-SAE are also detected by fluorescence after OPA derivatization but with a much lower response factor.

various parameters which govern the reversed-phase ion-pair chromatographic retention mechanisms, a satisfactory separation of the major SAM analogues and natural polyamines can be obtained. The use of 5-μm spherical packings has now greatly improved our separation. Fig. 1 shows the separation of a standard mixture of SAM analogues and some catechols and indoles, along with various polyamines and amino acids. The concentration of the anionic modifier, octanesulphonic acid, ionic strength of the buffer, column temperature, amount of acetonitrile and especially pH of the mobile phases were adjusted in order to obtain the desired separation[25]. Although the general separation pattern remained similar to the one previously obtained[17], the greater efficiency obtained with smaller diameter particles[26] markedly improves the separation. In the UV detection mode, as many as twelve compounds are clearly separated in the standard mixture, whereas with fluorescence detection after UV-cell derivatization with OPA fourteen amino acids and amines are analyzed in less than 30 min. Other amino acids or amines may be included in the separation by slightly changing the chromatographic conditions either by modifying the eluent composition or simply by using different gradients with longer times. As will be shown by the applications presented below, the conditions used have been found to be optimal.

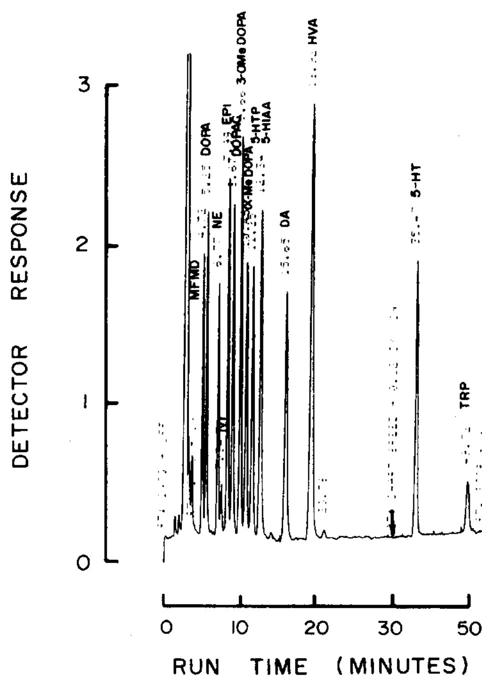

Fig. 2. Chromatogram of a standard solution of catechols and 5-hydroxyindoles. Amounts in pmol: MFMD (3.6); DOPA (3.8); NE (3.0); Tyr (19.0); EPI (4.7); DOPAC (4.2); 3-OMeDOPA (7.4); α-MeDOPA (4.9); 5-HTP (3.4); 5-HIAA (3.9); DA (4.2); HVA (8.8); 5-HT (8.1); Trp (21.0). Chromatographic conditions: column, Ultrasphere-IP, 5 μm (25 cm × 4.6 mm I.D.); electrode potential, +0.85 V *vs*. an Ag/AgCl reference electrode; temperature, 29.5°C; flow-rate, 1 ml/min; other conditions as indicated in Materials and methods. The arrow indicates the time at which the chart speed was reduced by a factor of 2.

TABLE II

CHROMATOGRAPHIC PROPERTIES OF THE VARIOUS CATECHOLS AND INDOLES DETERMINED BY ELECTROCHEMICAL DETECTION

Compound	*Retention time (min)*	*Detection limit* (pmol)*
MFMD	4.72	0.04
DOPA	5.25	0.04
NE	6.77	0.04
Tyr	7.34	1.3
EPI	7.99	0.04
DOPAC	8.67	0.04
3-OMeDOPA	9.66	0.06
α-MeDOPA	10.45	0.06
5-HTP	11.25	0.04
5-HIAA	12.34	0.04
DA	15.65	0.05
HVA	18.92	0.06
5-HT	35.47	0.09
Trp	49.72	1.3

* Expressed in pmol by considering a signal-to-noise ratio of 2. Chromatographic conditions as in Fig. 2.

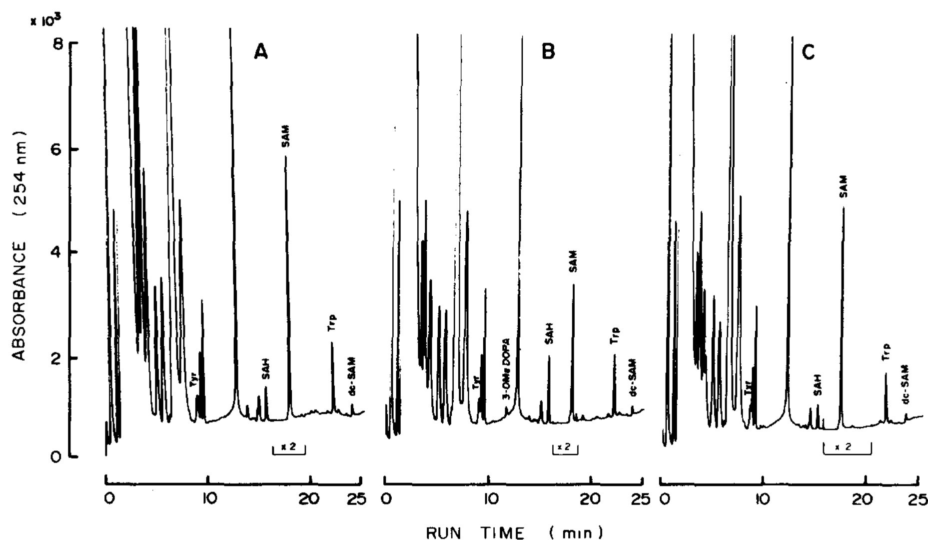

Fig. 3. Chromatograms of SAM derivatives with UV detection at 254 nm from rat brain: A, control; B and C, 2 h and 6 h after treatment with 100 mg/kg L-DOPA plus 1 mg/kg MFMD p.o. Chromatographic conditions as described in Materials and methods; a 20-μl aliquot of the clear supernatant after homogenization in 0.4 *M* $HClO_4$ and centrifugation was injected into the column. The sensitivity of the UV detector was set at 0.01 with the recorder scale at 10 or 20 mV (× 2).

The retention times of a number of SAM analogues, catechols, indoles, amino acids and polyamines are listed in Table I along with their detection limits. From these data it is obvious that neither the UV detection at 254 (or 280) nm nor the fluorescence detection with OPA derivatization are optimum methods for the analysis of the catechols and indoles[27]. The sensitivity for the polyamines, in the OPA fluorescence detection mode, is about twice that for the amino acids (see Table I).

Analysis of catechols and indoles. In previous papers[19,20] we showed that the use of an eluent of moderate ionic strength (0.1 *M*) and of octanesulphonic acid as an ion-pairing agent with a column of 10-μm particles allowed the simultaneous analysis of the major catechol and indole precursors and metabolites. Here too, the use of 5-μm spherical particles greatly improved the separation procedure. Fig. 2 shows the separation of a standard mixture of fourteen catechol and 5-hydroxyindole precursors and metabolites in amounts ranging from 0.2 to 1 ng using an Ultrasphere-IP column with 5-μm diameter particles and OSA as the ion-pairing agent. The pH, temperature and mobile phase composition were carefully adjusted to obtain the desired separation. The retention times and the detection limits are in Table II. The oxidation potential used, 0.85 V *versus* the silver–silver chloride reference electrode, allowed a satisfactory response for the 3-OMe analogues and for tryptophan and tyrosine without increasing too much the noise level of the baseline. Among the compounds not listed and of potential interest, 3,4-dihydroxyphenylglycol (DOPEG) and its O-methylated derivative (MOPEG) have retention times of 3.6 and 5.3 min respectively. They could not be measured in the tissue extracts under the conditions described. The sensitivity with detection limits in the low picogram range for the catechols and 5-hydroxyindoles is largely superior to those obtained by UV or flu-

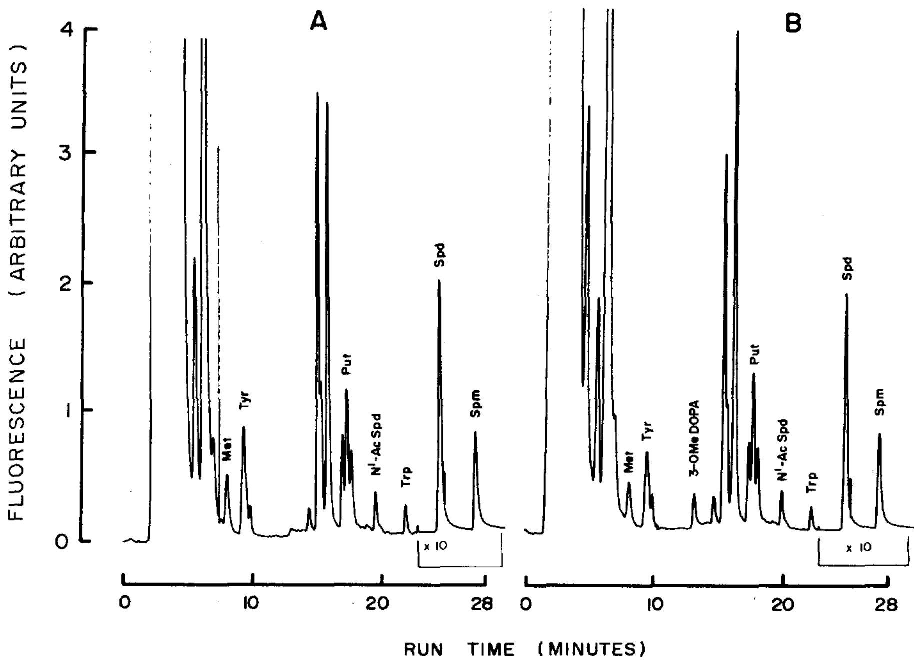

Fig. 4. Chromatograms of the polyamines and amino acids from rat brain; A, control; B, 2 h after treatment with L-DOPA plus MFMD. These chromatograms were obtained as described in Materials and methods during the same analyses as in Fig. 3 after UV-cell derivatization with OPA and subsequent fluorescence detection. At × 10, the attenuation of the recorder was increased by a factor of 10.

orescence detection after OPA derivatization, and are similar to those reported in the literature. They made HPLC with electrochemical detection the method of choice for the analysis of the catechols and 5-hydroxyindoles[28-31].

Applications to rat tissue analysis

Time-course effect of a single dose of DOPA with and without MFMD. MFMD has been shown to be a potent enzyme-activated irreversible inhibitor of AADC[21,22]. It was therefore of interest to study the effects of a treatment with both L-DOPA and MFMD on the levels of SAM and its analogues as compared to the treatment with L-DOPA alone.

The time-course effect of a single dose of L-DOPA, 100 mg/kg, administered by gavage (p.o.) alone and with MFMD, 1 mg/kg, was studied by monitoring the levels of SAM and its analogues along with the polyamines in various tissues of rats. Concurrently, the catechols and indoles were analyzed in the same tissue extracts by using the HPLC procedure with electrochemical detection. Fig. 3 shows typical chromatograms obtained by direct injection of the perchloric acid extracts of rat brain samples and UV detection at 254 nm. SAH, SAM and dc-SAM along with Tyr and Trp are easily determined in the control sample (A). Two hours after the combined treatment with DOPA and MFMD (B), the peak corresponding to SAM is decreased whereas SAH is increased and 3-OMeDOPA is now observed. Six hours after treatment (C), the SAM peak starts to return towards its control level whereas SAH is

TABLE III

TIME-COURSE EFFECT OF A SINGLE DOSE OF L-DOPA (100 mg/kg p.o.) OR L-DOPA PLUS MFMD (MFMD 1 mg/kg p.o.) ON BRAIN AND HEART CONCENTRATIONS OF SAM, SAH AND 3-OMeDOPA

The values are expressed in nmol per g wet weight ± S.E.M. (n = 5). ND = Not detectable.

Treatment		*Brain*			*Heart*		
		SAH	*SAM*	*3-OMeDOPA*	*SAH*	*SAM*	*3-OMeDOPA*
Control		0.77 ± 0.03	19.9 ± 0.7	ND	0.77 ± 0.05	41.9 ± 1.8	ND
L-DOPA	1 h	1.06 ± 0.04	14.3 ± 0.4	4.9 ± 0.6	1.82 ± 0.13	16.6 ± 0.80	6.2 ± 1.0
alone	2 h	1.05 ± 0.06	12.7 ± 0.9	10.1 ± 0.3	1.60 ± 0.13	14.5 ± 1.5	12.3 ± 0.9
	4 h	0.69 ± 0.03	18.1 ± 0.5	9.6 ± 0.3	0.95 ± 0.06	34.6 ± 1.0	15.0 ± 1.2
	6 h	0.72 ± 0.05	20.2 ± 0.5	9.8 ± 1.0	0.97 ± 0.04	44.6 ± 1.7	10.9 ± 0.9
L-DOPA	1 h	1.90 ± 0.4	12.5 ± 0.5	6.4 ± 0.9	2.38 ± 0.14	14.4 ± 0.4	7.9 ± 0.7
+	2 h	2.12 ± 0.12	10.6 ± 0.5	20.5 ± 5.6	2.38 ± 0.11	11.6 ± 0.7	18.1 ± 1.0
MFMD	4 h	1.86 ± 0.04	9.7 ± 0.4	41.5 ± 3.7	2.49 ± 0.07	12.5 ± 0.2	50.4 ± 3.9
	6 h	0.85 ± 0.04	16.1 ± 0.4	46.6 ± 4.1	1.69 ± 0.13	28.3 ± 1.4	63.1 ± 4.0

decreasing. Dc-SAM, Tyr and Trp do not show any marked changes. Fig. 4 presents the chromatograms obtained by the post-UV derivatization procedure with OPA for the rat brain control sample (A) and 2 h after the combined DOPA–MFMD treatment (B). Met, Tyr, Put, N^1-AcSpd, Trp, Spd and Spm are clearly detected in the control sample (Fig. 4A). Two hours after treatment (Fig. 4B), 3-OMeDOPA is detected and the peak corresponding to methionine slightly decreases, whereas no changes are observed for the other compounds. Similar chromatograms were obtained for samples of heart, pancreas and ventral prostate tissues of the same rats. Whereas in the chromatograms of the heart samples the same variations in the levels of SAM and SAH were observed as for the brain samples, the chromatograms of the pancreas and prostate samples following treatment did not indicate any marked changes either in the UV or in the fluorescence detection mode. The chromatograms obtained for the same tissue extracts by the HPLC procedure using electrochemical detection exhibited similar patterns and variations to those already described[20]. Table III summarizes the time-course effect of DOPA alone and with MFMD on the main biochemical markers involved, *i.e.*, SAM, SAH and 3-OMeDOPA, in the brain and in the heart, since no sizeable variations, at least in the levels of SAM and SAH, were observed in prostate and pancreas. It is, however, worth mentioning that the catechols, *i.e.*, DOPA and 3-OMeDOPA, showed, in prostate and pancreas, similar variations to those observed in brain and heart. The results are expressed in nmol per g tissue ± S.E.M. after correction for recovery.

The values obtained for SAH and SAM in the brains of control rats, *i.e.*, 0.8 and 20 nmol/g respectively, are in good agreement with some published values[12,32,33] although lower or higher values for SAM have been reported[34–36]. The corresponding values obtained in the hearts of control rats, 0.8 and 42 nmol/g respectively, are within the range of published data[12,33] although our values for SAH tend to be slightly lower.

The results clearly confirm the potency of MFMD as an AADC inhibitor and are similar to those already obtained[37] for DOPA and 3-OMeDOPA. At 6 h, the

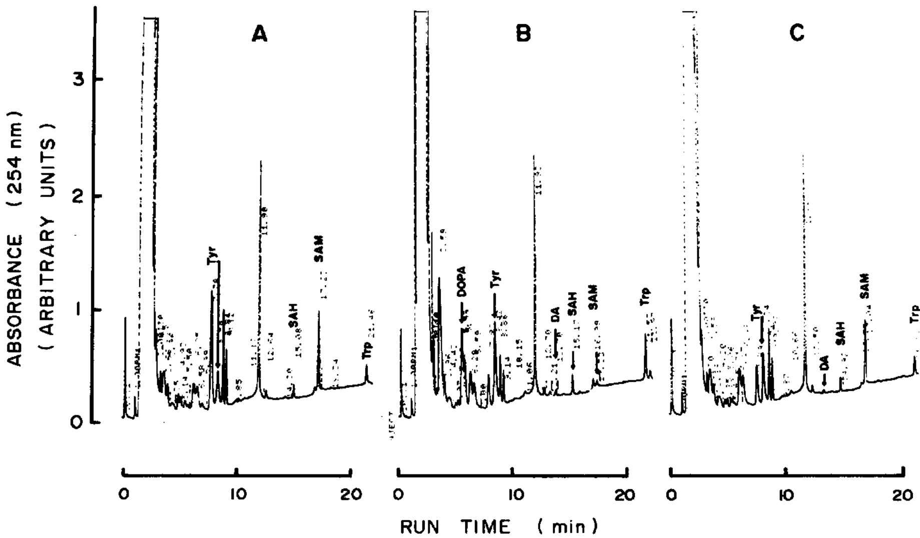

Fig. 5. Chromatograms obtained with UV detection at 254 nm of rat adrenals: A, control; B and C, 2 h and 8 h after chronic administration of L-DOPA (100 mg/kg) and MFMD (1 mg/kg) p.o. Chromatographic conditions as described in Materials and methods and in Fig. 1. An 80-μl aliquot of the adrenal extracts (4 ml) was injected.

levels of 3-OMeDOPA are five to six times higher after the combined treatment than following treatment with DOPA alone. The SAM levels exhibit similar decreases between 1 and 4 h after treatment, with DOPA alone or with DOPA plus MFMD, although the combined treatment shows a longer lasting effect (for example, see differences at 4 h). The increase in SAH levels is more pronounced in brain and heart after the combined treatment. As already observed following treatment with DOPA

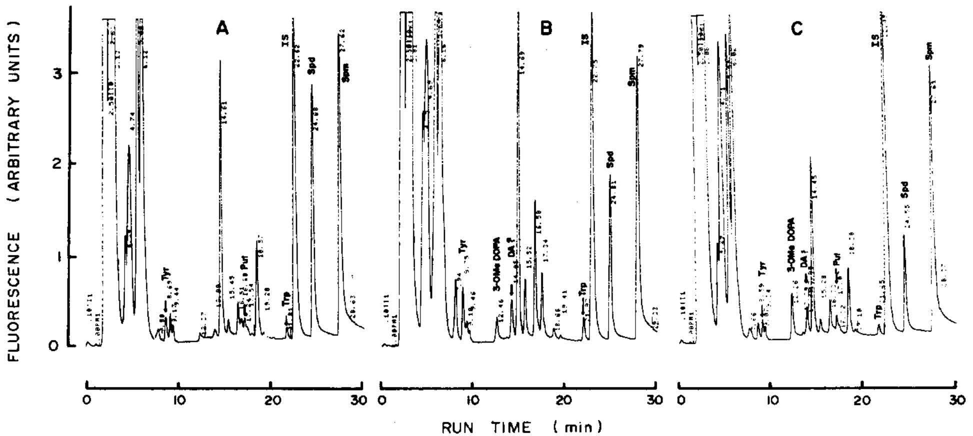

Fig. 6. Chromatograms of the polyamines and amino acids from rat adrenals: A, control; B and C, 2 h and 8 h after chronic administration of L-DOPA (100 mg/kg) and MFMD (1 mg/kg) p.o. These chromatograms were obtained during the same analysis as in Fig. 5 after UV cell derivatization with OPA. The chromatographic conditions were as described in Materials and methods and in Fig. 1.

alone[4,5], this decrease in SAM level is probably related to an increased consumption of SAM by the COMT enzyme and to a rate-limiting synthesis of SAM by SAM synthetase.

Chronic treatment with both DOPA and MFMD. In order to study the cumulative effects of a chronic treatment which would more closely reflect the clinical situation of L-DOPA coadministered with an AADC inhibitor, L-DOPA (100 mg/kg) with MFMD (1 mg/kg) was administered to rats by gavage (p.o.) every 12 h during 5 days. The rats were sacrificed 2, 4, 8 and 18 h after the last dose and the different tissues, *i.e.*, brain, heart, ventral prostate, pancreas, kidney and adrenals, were removed and analyzed by using the two HPLC procedures described. Fig. 5 shows the chromatograms obtained by UV detection at 254 nm for the adrenal extracts of control rats treated with saline (A), and 2 h (B) and 8 h (C) after the last dose of L-DOPA–MFMD. SAH and SAM are clearly detected in the control sample (A), whereas 2 h after treatment the SAM peak is considerably decreased and that of SAH is increased (B). At 8 h (C), SAM and SAH have returned towards their control levels. Tyr and Trp show a slight increase 2 h after treatment (B). Fig. 6 presents the corresponding chromatograms obtained by fluorescence detection. Tyr, Put, Trp, Spd and Spm are easily detected in the control sample (A) along with 1,7-diaminoheptane used as internal standard (IS). After treatment, 3-OMeDOPA is observed (Fig. 6B and C) whereas Tyr and Trp exhibit the same increase 2 h after treatment as displayed in Fig. 5. Put, Spm and Spd seem to decrease slightly after treatment (see Fig. 6B and C) but the variations are not significant (see above). The other tissue samples were analyzed using the same chromatographic procedure. The chromatograms of brain, heart and to a lesser extent kidney indicated similar variations for SAM and SAH to those observed for adrenals, whereas no changes in the levels of SAM and SAH were observed for the pancreas and prostate. The HPLC analysis with electrochemical detection of the same extracts gave similar chromatograms to those already reported[20], with pronounced increases in the peaks of DOPA, 3-OMeDOPA and to a lesser extent of DOPAC and HVA.

The most interesting results obtained in brain, adrenals and pancreas are summarized in Table IV which shows the variations of SAM, SAH and of the major catechols and their metabolites, DOPA and 3-OMeDOPA, DOPAC and HVA, NE, EPI and DA as a function of time after the last treatment with L-DOPA–MFMD. The polyamine levels did not show any marked variations (data not shown). The amounts of the polyamines in the tissues of control rats were overall in good agreement with published data[38].

The amounts of SAH and SAM in the brains of control rats, 0.8 and 18 nmol/g respectively, show good reproducibility. The values found for SAH and SAM, 6 and 48 nmol/g for the adrenals, 7 and 31 nmol/g for the pancreas, are in good agreement with published results[14,34,35]. The levels of the various catechols in the different tissues of control rats were similar to those published[20].

Although the variations of SAM and SAH in the brain and the adrenals show some similarities, the decrease in the level of SAM is much more pronounced at 2 h in the adrenals than in the brain (15% of its control level in adrenals *versus* 65% of the control level in brain at this time). This decrease in SAM levels is accompanied by a nearly two-fold increase in the SAH levels at 2 h in both tissues. At 4 h, SAM and SAH return towards their control levels although the DOPA levels remain mark-

TABLE IV

EFFECT OF CHRONIC TREATMENT WITH L-DOPA COMBINED WITH MFMD ON SAH, SAM AND CATECHOL LEVELS IN VARIOUS TISSUES OF RATS

ND = Not detectable. Values are expressed in nmol per g wet weight ± S.E.M. (n = 5). Rats were treated with 100 mg/kg L-DOPA + 1 mg/kg MFMD p.o., coadministered every 12 h during 5 days. The animals were killed 2, 4, 8 and 18 h after the last administration. The control animals received an equal volume of vehicle and were sacrificed 2 h after the last treatment.

Tissue	*Treatment*	*SAH*	*SAM*	*DOPA*	*3-OMeDOPA*	*DOPAC*	*HVA*	*NE*	*DA*
Brain	Control	0.80 ± 0.05	17.9 ± 0.4	0.3 ± 0.1	0.4 ± 0.05	0.8 ± 0.05	0.5 ± 0.05	2.1 ± 0.2	5.2 ± 0.2
	2 h	1.40 ± 0.05	11.7 ± 0.3	28 ± 2	58 ± 5	5.6 ± 0.4	5.2 ± 0.4	1.8 ± 0.1	10.2 ± 0.2
	4 h	1.10 ± 0.05	12.9 ± 0.3	21 ± 4	91 ± 15	3.3 ± 0.3	3.6 ± 0.4	2.1 ± 0.1	8.9 ± 0.6
	8 h	0.85 ± 0.05	16.7 ± 0.7	8 ± 1	89 ± 9	1.5 ± 0.2	1.7 ± 0.1	2.5 ± 0.1	6.8 ± 0.2
	18 h	0.80 ± 0.05	20.5 ± 0.4	0.3 ± 0.1	33 ± 1	0.7 ± 0.2	0.4 ± 0.05	2.2 ± 0.11	4.8 ± 0.2
Adrenals	Control	6.2 ± 0.45	47.9 ± 4.7	ND	ND	ND	ND	525 ± 73	26 ± 3
	2 h	10.8 ± 0.70	7.3 ± 0.8	19 ± 2	100 ± 8	ND	ND	605 ± 36	134 ± 14
	4 h	9.3 ± 1.1	34.9 ± 2.8	39 ± 7	158 ± 31	ND	ND	685 ± 90	139 ± 27
	8 h	7.1 ± 0.4	43.8 ± 2.6	12 ± 2	136 ± 13	ND	ND	745 ± 90	79 ± 7
	18 h	6.7 ± 0.6	46.0 ± 5.2	4 ± 1	61 ± 4	ND	ND	508 ± 89	20 ± 3
Pancreas	Control	6.7 ± 0.7	30.8 ± 1.4	0.01 ± 0.01	1.2 ± 0.1	⩽ 0.1	⩽ 0.2	2.4 ± 0.2	0.4 ± 0.1
	2 h	7.5 ± 0.3	30.7 ± 1.3	280 ± 2	411 ± 24	4.7 ± 1.3	4.3 ± 1.6	3.2 ± 0.1	4.2 ± 0.7
	4 h	8.3 ± 0.7	27.8 ± 1.2	218 ± 41	711 ± 134	4.2 ± 0.8	3.1 ± 0.6	2.9 ± 0.2	3.6 ± 0.4
	8 h	7.3 ± 0.7	29.7 ± 1.7	88 ± 17	708 ± 72	3.6 ± 0.9	1.7 ± 0.4	2.9 ± 0.1	4 ± 0.6
	18 h	7.5 ± 0.5	35.0 ± 2.2	1 ± 0.2	255 ± 10	0.4 ± 0.1	0.4 ± 0.1	1.9 ± 0.2	1.7 ± 0.4

edly elevated. It seems that the SAM level is only markedly decreased when the rate of O-methylation of DOPA to 3-OMeDOPA is maximal and this occurs between 1 and 2 h as can be deduced from Table IV.

The decrease in the level of SAM observed in brain after chronic treatment with DOPA plus MFMD (Table IV) does not differ markedly from the one observed after a single dose (see Table III). This suggests that the DOPA levels reached after a single and a chronic treatment of DOPA plus MFMD are similar, or that the activity of SAM synthetase is increased by a chronic treatment. This is not really unexpected since the levels reached by DOPA after a single dose, although lower at 2 h (9 *versus* 28 nmol/g), are quite similar at 4 h (22 *versus* 21 nmol/g). The slight rebound exhibited by the SAM level at 18 h in the brain (Table IV) is probably due to an increased activity of SAM synthetase. From these results, it is clear that the SAM level decreases when the rate of O-methylation is higher than the rate of synthesis of SAM. It is well known that the activity of SAM synthetase varies greatly from one tissue to another[33]. The activities in brain, heart and adrenals were found to be 43, 42 and 80 pmol/min per mg of protein respectively, whereas in pancreas, prostate and kidney, values of 558, 141 and 192 pmol/min respectively, were reported[33]. Furthermore, the relative activities of COMT in the same tissues also vary greatly from 0.1, 0.2, 2.1 to 6.3 μmol metanephrine formed per g tissue per h for heart, brain, kidney and adrenals respectively[39]. The relative activities of COMT and SAM synthetase probably account for the differences observed in the variations of SAM and SAH in the different tissues studied. In this respect, although DOPA and 3-OMeDOPA reach their maximum concentrations, as expressed in nmol per g tissue, in the pancreas (Table IV), no diminution in the level of SAM is observed since the SAM synthetase activity is highest in this tissue. This result is to be compared with the pronounced decrease in the level of SAM in adrenals where the COMT activity is greatest and the SAM synthetase activity is relatively low.

CONCLUSIONS

The combination of UV absorbance detection and OPA derivatization allows the determination in a single analysis and in less than 45 min of the major SAM analogues and natural polyamines. The procedure may be further varied or improved by changing the gradient profile and duration, but at the expense of an increased analysis time. The use of 5-μm packings with standard size columns (25 cm × 4.6 mm) has greatly improved the separation efficiency. Both methods involve simplified sample preparation by direct injection of the clear supernatant after tissue homogenization and centrifugation, without adverse effects on the column lifetime. Some columns have been in use for more than 1 year and over 3000 samples have been analyzed without marked loss in performance. It is expected that the use of still smaller diameter packings (3-μm diameter particles) and of smaller size columns will further improve the efficiency of the separation and greatly reduce the analysis time.

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