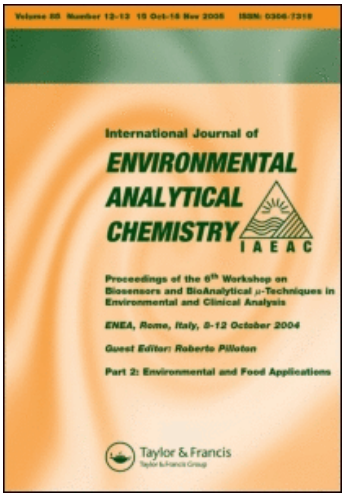

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Separation of Twenty Biogenic Amines and Derivatives by a High-performance Liquid Chromatographic Column Switching Technique with On-line Fluorimetric and Electrochemical Detections†

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A new high-performance liquid chromatographic technique including the use of an automated column switching system has been developed for the study of dopamine, norepinephrine, epinephrine and serotonin and their related metabolites in biological samples. Through two runs, it has been possible to separate twenty derivatives and three internal standards which have to be added to samples prior to the extraction procedures. In each case, the column switching system allowed to obtain a clear separation of all the compounds, which will be of importance to avoid any expected interference from other endogenous substances, while decreasing the analysis time. By coupling on-line fluorimetric and electrochemical detections the specificity of the technique was enhanced, since the ratio of the responses of both detectors was an index of the purity of the peaks. In addition, fluorimetric detection was found of value

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to free the determination of some compounds from the effects of solvent front while electrochemical detection increased the sensitivity. Finally, the column switching system allowed a rapid cleaning of the columns between two analyses. A typical application to a human urine sample was shown.

KEY WORDS: Biogenic amines, HPLC separation, column switching.

INTRODUCTION

The catecholamines, epinephrine, norepinephrine and dopamine are of demonstrated biological importance for the diagnosis and management of clinical disorders and for the study of pathophysiological mechanisms, while serotonin and related compounds have been strongly implicated in a number of neural disorders. Since biogenic amines undergo complex metabolic pathways, a precise knowledge of the neuronal activity requires the measurement of all the known metabolites of these neuromediators (Fig. 1).

Thus in essential hypertension, urinary catecholamines and their metabolites might reflect a sympathetic overactivity which has been thought to play a significant role in both initiation and maintenance of hypertension.[1]

Several techniques using high-performance liquid chromatography (HPLC) with fluorimetric[2,3] (FD) or electrochemical[4-9] (ECD) detection have been developed for the measurement in different tissues[10-12] or fluids[13-16] of catecholamines and/or metabolites. However each of these methods is devoted to the measurement of a small number of catecholamines and metabolites.

In addition, if it has been possible to quantify VMA,[17,18] HVA,[11,8] DOPAC,[11,19] MOPEG,[20-22] NMN, MN and MeDA,[23,24] there is little information on other metabolites such as DOMA and DOPEG,[25,26] DOPET, MOPET, or precursors, DOPA and MeDOPA.

Therefore we thought it was interesting to develop a new analytical procedure which could allow the separation and the quantification of the 16 catecholamines, precursors and metabolites as well as serotonin and its derivatives, in order to obtain a precise knowledge of the biosynthesis and inactivation of biogenic amines in humans and in animals.

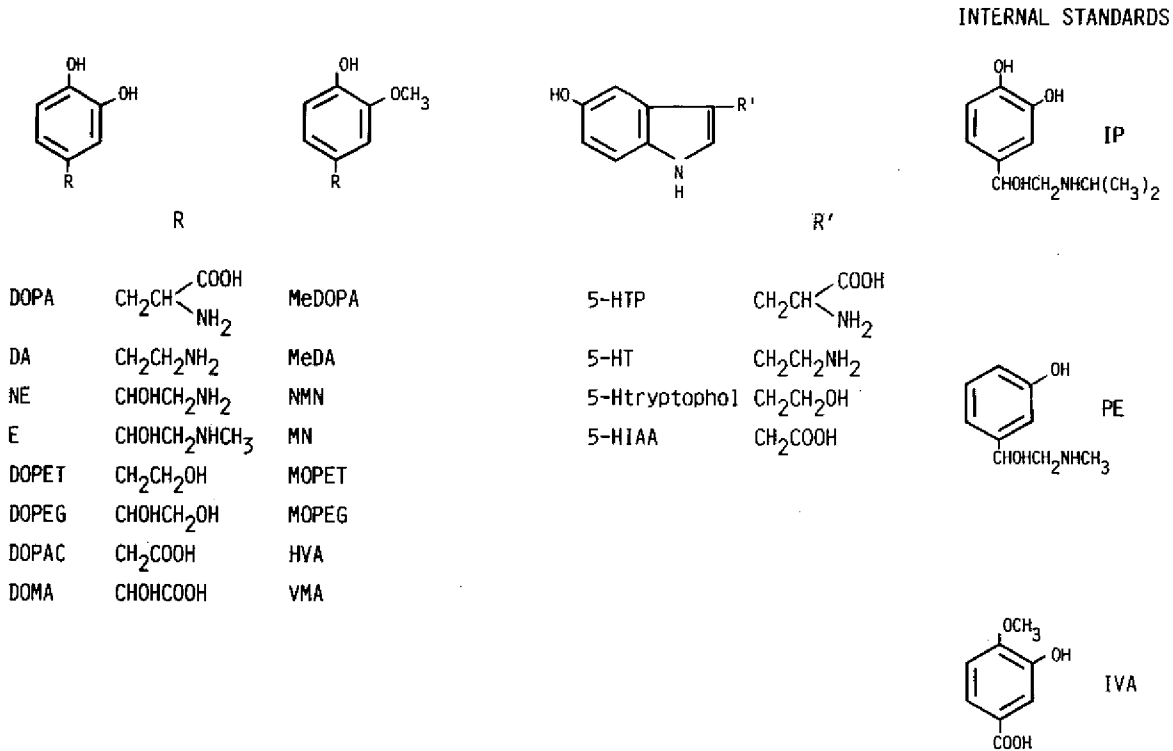

FIGURE 1 Catecholamines, serotonin, precursors and their related metabolites issued from catabolic pathways; isoproterenol (IP), phenylephrine (PE) and isovanillic acid (IVA) as possible internal standards.

This was achieved by using a new automated column switching HPLC system and by coupling fluorimetric and electrochemical detections.

MATERIALS AND METHODS

Apparatus

The whole HPLC system (Fig. 2) was constituted as follows:

—A Kontron liquid delivery system (Kontron, Zurich, Switzerland) equipped with a high-pressure pump, Model 410 and pulse damper, Model 811; the solvent reservoir was kept in a thermostated water-bath set at 22°C ± 0.1.

—A Kontron column switching system, Tracer, Model MCS 670, with a loop injector and four stainless-steel columns the commutation of which (valve switching configuration is shown in Fig. 3) was monitored by a microprocessor controlled programmer, Tracer Timer, Model MCU 210.

—A Kontron fluorescence sensitive detector, Model SFM 23, set at 282 nm excitation wavelength and at 314 nm emission wavelength (excitation and emission slits, 4 nm).

—A Brucker electrochemical detector (Brucker Spectrospin, Bruxelles, Belgium), Model E 230 equipped with a thin-layer flow-through cell, Model LCC 231, with glassy carbon as the working and auxiliary electrodes set at a potential of +0.72 V versus the calomel reference electrode.

—A two channel recorder (W+W Electronic Inc., Basel, Switzerland), Model 600, set at 50 mV and 1000 mV full scale for fluorimetric and electrochemical detectors respectively. Recorder pens were shifted on the paper as if there was a 5 min. time space between them when the recorder speed was set at 12 cm/h.

Chemicals and reagents

Epinephrine (E), norepinephrine (NE), dopamine hydrochloride (DA), 3, 4-dihydroxyphenylalanine (DOPA), 3, 4-dihydroxymandelic acid (DOMA), 3, 4-dihydroxyphenylethyleneglycol (DOPEG), 3, 4-dihydroxyphenyl-acetic acid (DOPAC), metanephrine hydrochloride (MN), normetanephrine hydrochloride (NMN), 3-methoxy-4-

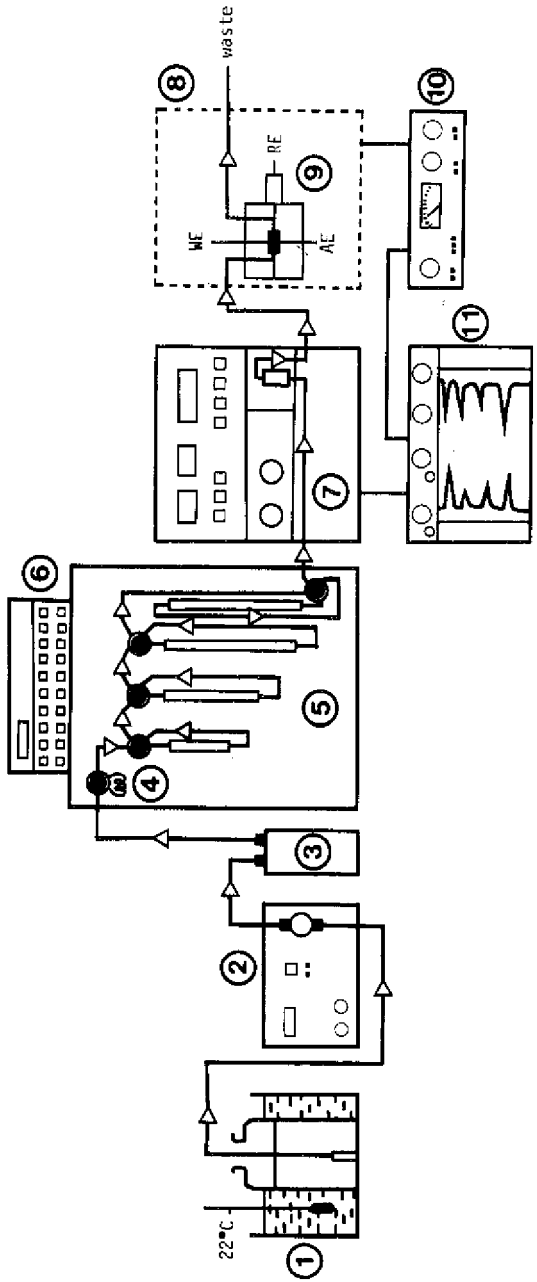

FIGURE 2 Schematic diagram of the liquid chromatography system: 1=solvent reservoir, 2=pump, 3=pulse damper, 4=loop injector, 5=column switching system, 6=time programmer, 7=fluorimetric detector equipped with an 8 μl cell, 8=Faraday cage, 9 =15 μl thin layer electrochemical cell (W.E. and A.E.=working and auxiliary glassy carbon electrodes respectively; R.E.=calomel reference electrode), 10=electrochemical detector, 11=two channel recorder.

hydroxyphenylalanine (MeDOPA), 3-methoxy-4-hydroxyphenylethylamine hydrochloride (MeDA), vanilmandelic acid (VMA), bis(3-methoxy-4-hydroxyphenylethyleneglycol) piperazine salt (MOPEG), homovanillic acid (HVA), 5-hydroxytryptophan (5-HTP), 5-hydroxytryptamine hydrochloride (5-HT), 5-hydroxytryptophol (5-Htryptophol), 5-hydroxyindolacetic acid (5-HIAA), isovanillic acid (IVA), isoproterenol hydrochloride (IP) and phenylephrine hydrochloride (PE) were purchased from Sigma, St. Louis, M.O., U.S.A.; 3, 4-dihydroxyphenylethanol (DOPET) was obtained from Serva, Heidelberg, G.F.R., and 3-methoxy-4-hydroxyphenylethanol (MOPET) from Aldrich, St. Regis, W.I., U.S.A. These compounds were used without further purification.

All other reagents were of analytical grade: potassium dihydrogen phosphate, KH_2PO_4 (Merck, Darmstadt, G.F.R.); sodium metabisulfite, $Na_2S_2O_5$ (Merck); ethylenediaminetetracetic acid disodium salt, Na_2-EDTA (Carlo-Erba, Milano, Italy); tris(hydroxymethyl)amino-methane, Tris (Merck); sodium octyl sulfate (SOS) (Merck) used as ion pairing agent. Methanol (Prolabo, Paris, France) was distilled before use.

The mobile phase consisted of 0.06 M KH_2PO_4, 50 mg/l Na_2-EDTA, 100 mg/l SOS and 12.5% (v/v) of methanol. This mixture was adjusted to pH = 3.5 ± 0.03 at 22°C with 15 N phosphoric acid.

Stock solutions of biogenic amines, derivatives and internal standards were monthly prepared by dissolving 100 μg/ml of each compound (calculated as free compounds for salt-complexed derivatives) in 0.3 N phosphoric acid containing 0.1% of $Na_2S_2O_5$. Working standard solutions were weekly diluted from their corresponding stock solutions in the same solvent in order to obtain concentrations between 10 ng/ml and 10 μg/ml. All these solutions were stored at 4°C in darkness.

Chromatographic technique

All the columns were packed with Nucleosil C18, 10 μm (Macherey–Nagel, Düren, G.F.R.) by the upward slurry packing technique as described by Bristow *et al.*,[27] with methylisobutylketone as the slurry medium and dichloromethane as the follower. All stainless-steel column parts were cleaned by treating them successfully with dichloromethane, acetone, 5 M nitric acid, acetone and

dichloromethane again in an ultrasonic bath for 5 min. The slurry concentration was approximately 4% (w/v) and the applied pressure 350–400 bar. After packing, the support was purified by pumping about 100 ml methanol at a 2 ml/min flow-rate through the column. Column performance was tested according to Bristow and Knox.[28]

Mobile phase was degassed in vacuum for 10 min. and deaerated by bubbling through oxygen-free nitrogen for 10 min. Column equilibrium was achieved within 10–15 h at a 1 ml/min flow-rate. A 0.1 ml/min flow-rate was continuously maintained through all the columns during the standby periods (day and night).

Resolution between two consecutive peaks was calculated as

$$R_s = 2(t_{r2} - t_{r1})/(w_1 + w_2)$$

where t_{r1} and t_{r2} are the uncorrected retention times of components 1 and 2, and w_1 and w_2 are their basal peak widths. Peak symmetry was measured at a distance from the baseline of 10% of the peak height. All chromatographic values were the mean of duplicate or triplicate determinations.

Description and use of the column switching system

As shown in Fig. 3, this apparatus consisted of:

—one injection valve V (Rheodyne, Cotati, C.A., U.S.A.), Model 7125 with a 100 μl loop;

—four valves (Rheodyne), Model 7010, controlled with an electro-pneumatic unit monitored by an external programmer or an internal keyboard; the use of pneumatic cylinders to control these valves (V1 to V4) gave a fast switch over and helped to ensure a good repeatability of commutation;

—four chromatographic columns (C1 to C4) were connected in 3–6 ports of each corresponding valve; their length were 10, 25, 25 and 25 cm for C1, C2, C3 and C4 respectively;

—all the connections were made of 0.25 mm internal diameter stainless-steel tubing in order to minimize the dead volumes between valves and columns.

Using the different commutation possibilities several configurations of the effluent flow route could be obtained:

—in the standby position (without column) so indicated by dark arrows in Fig. 3, the mobile phase flowed from the pump through

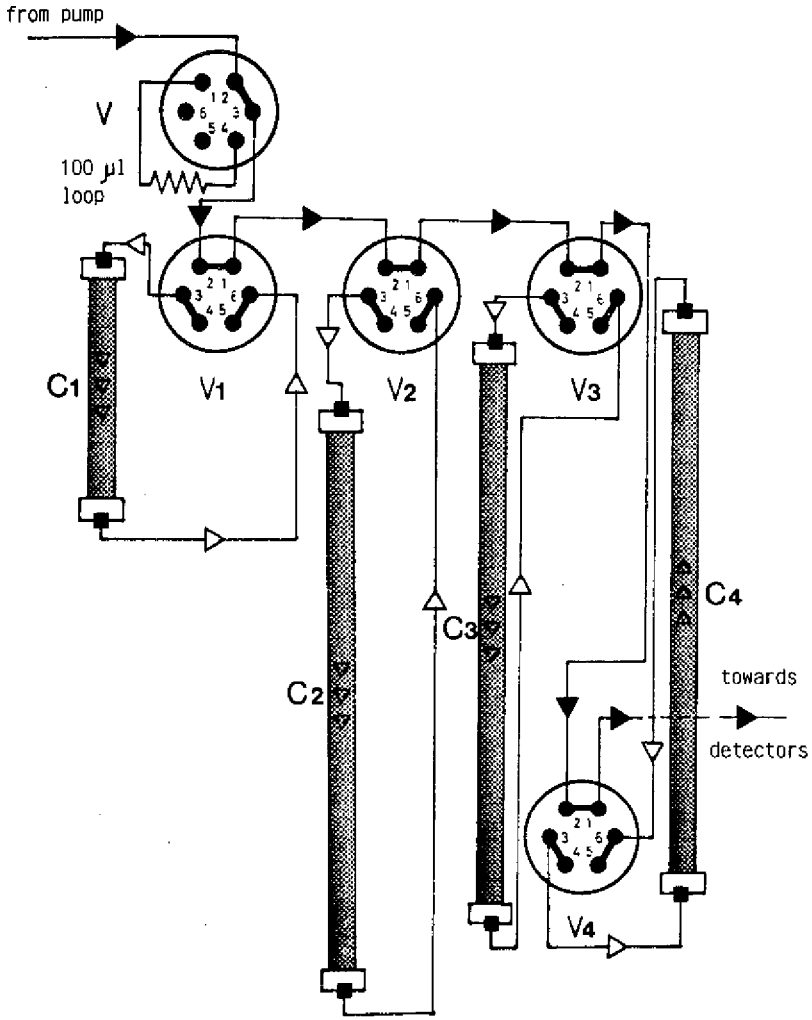

FIGURE 3 Configuration of the column switching apparatus: V = injection valve; V1, V2, V3 and V4 = commutation valves; C1, C2, C3 and C4 = chromatographic columns (I.D. = 4.6 mm) of 10, 25, 25 and 25 cm of length respectively.

the positions 2–3 of the injection valve then the positions 2–1 of each other valve (V1 to V4) towards detectors;

—in the working position after passage through the injection loop when any one of the columns had to be used the corresponding valve was commuted so as the effluent flowed through the positions 2–3–6–1 of the valve as indicated with open arrows in Fig. 3.

As a consequence, at any moment of the analysis, the compounds of the injected sample could be chromatographed throughout any length of column as if there was a "gradient of stationary phase".

RESULTS AND DISCUSSION

According to their structural analogy (Fig. 1) and the foreseen extraction modes, the compounds were divided as follows:

—groupe I: catechol derivatives ($n=8$) with IP as possible internal standard to account for the classical alumina extraction procedure[29] used for these compounds;

—groupe II: methoxylated derivatives ($n=8$), serotonin and its derivatives ($n=4$) with PE and IVA as possible internal standards which could be used to account for the ion-exchange resin extraction for amine derivatives ($n=6$) and the organic solvent extraction for acidic and neutral compounds ($n=6$) respectively.

Choice of chromatographic support

Commercially available supports with varying chromatographic performances were tested (preliminary studies) in order to find the most suitable material.

Two (Lichrosorb RP 18, 10 μm and 5 μm, Merck) among the studied supports gave very low efficiencies and severe tailing peaks, while only Nucleosil C18, 10 μm and 5 μm were of high performance and with spherical particles, these offered the advantage to hold out against the sudden pressure changes induced by the column switching system. The efficiency and peak symmetry obtained with Nucleosil C18, 10 μm were slightly inferior to that given by Nucleosil C18, 5 μm. However, the former support was chosen because it dealt lower solvent pressure. Thus, the total length of column ($l=85$ cm) gave a maximal pressure of 250 bar with a 1.5 ml/min flow-rate.

Choice of mobile phase

Its composition was determined according to the classical mobile phases described by others[5,6,30] for this kind of analysis. The influence of pH, addition of methanol and ion-pairing agent was checked and the results were in total agreement with those previously reported.

Briefly, the retention of acidic compounds decreased with increasing pH, while the amines tended to be more strongly retained at higher pH and the neutral derivatives were not affected by this factor. Addition of the organic solvent to the eluting solvent decreased the retention time of all the compounds and did not alter the order of elution. The addition of SOS, as an ion-pairing agent increased the retention of amines while leaving acid and alcohol metabolites relatively unaffected so that the elution order could be manipulated.

In the present study, the pH was fixed at 3.5 ± 0.03, so as to increase the retention of DOMA, DOPA, VMA and MeDOPA, the most rapidly eluted compounds. The addition of 12.5% of methanol allowed to reduce the time of the run without affecting the separation of the compounds and 100 mg/l of SOS were sufficient to ensure the separation of some amines (NE, E, NMN, 5-HT and MN) by an ion-pairing effect.

Separation of the compounds

For both groups, the total length of column (4 columns, $l = 85$ cm) was needed to ensure a high separation of the most rapidly eluted compounds (DOMA, DOPEG, DOPA and VMA, MOPEG, MeDOPA for groups I and II respectively).

As shown in Fig. 4 and 5 for group I and Fig. 6 and 7 for group II, typical chromatograms of the mixtures were obtained without (Mode I) and with (Mode II) the use of the column switching system (CSS). For each group, a good separation of the compounds was obtained in both elution modes, however the total analysis times were 70 and 100 min. in Mode I and only 40 and 70 min. in Mode II for groups I and II respectively.

The important decrease in the duration of the analysis was achieved:

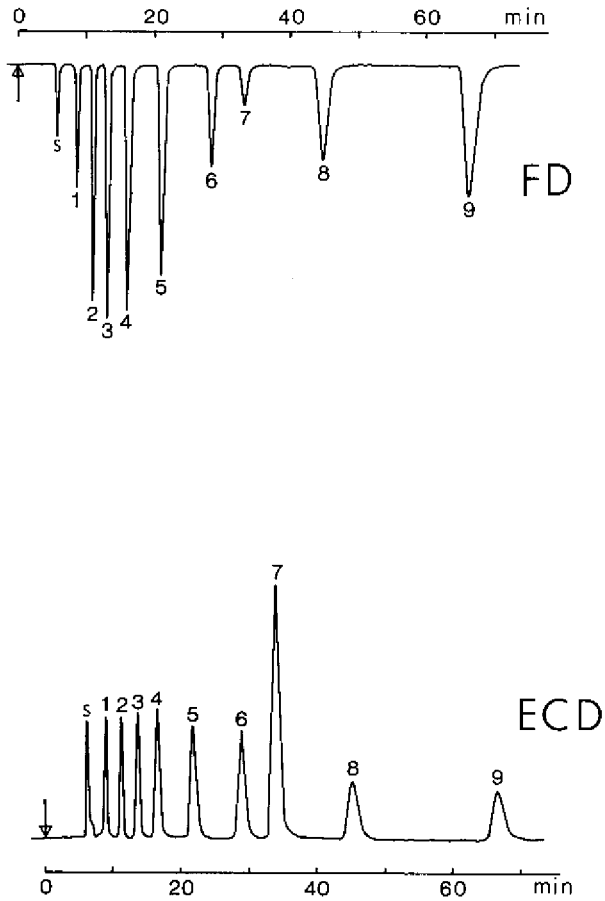

FIGURE 4 Chromatogram of a mixture of catechol derivatives (group I) and internal standard (IP) without the use of the column switching system: s = solvent, 1 = DOMA, 2 = DOPEG, 3 = DOPA, 4 = NE, 5 = E, 6 = DOPET, 7 = DOPAC, 8 = DA, 9 = IP. (FD = fluorimetric detection; ECD = electrochemical detection).

—For group I by eluting DOMA, DOPEG, DOPA, NE and E through 4 columns, DOPET and DOPAC through 3 columns (1 = 60 cm), DA through 2 columns (1 = 35 cm) and IP through only the first column (1 = 10 cm) according to the changes of column indicated by the arrows in Fig. 5. The choice of these changes was determined by considering the retention time of each compound and

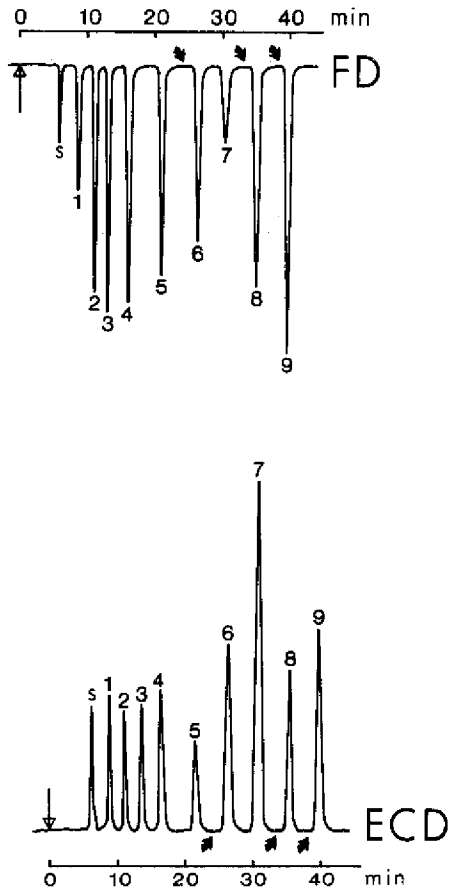

FIGURE 5 Chromatogram of a mixture of catechol derivatives (group I) and internal standard (IP) with the use of the column switching system: solvent (s), DOMA (1), DOPEG (2), DOPA (3), NE (4) and E (5) were eluted through 4 columns (1 = 85 cm), DOPET (6) and DOPAC (7) through 3 columns (1 = 60 cm), DA (8) through 2 columns (1 = 35 cm) and IP (9) through the first column (1 = 10 cm); the arrows indicate the column switching. (FD = fluorimetric detection; ECD = electrochemical detection).

the resolution between two consecutive peaks obtained after elution through each column configuration (Table I). It appeared that the resolution between DA and IP ($R_s = 3.11$), between DOPAC and DA ($R_s = 3.27$) and between E and DOPET ($R_s = 3.55$) was sufficient ($R_s > 3$) to allow the commutation of the corresponding column.

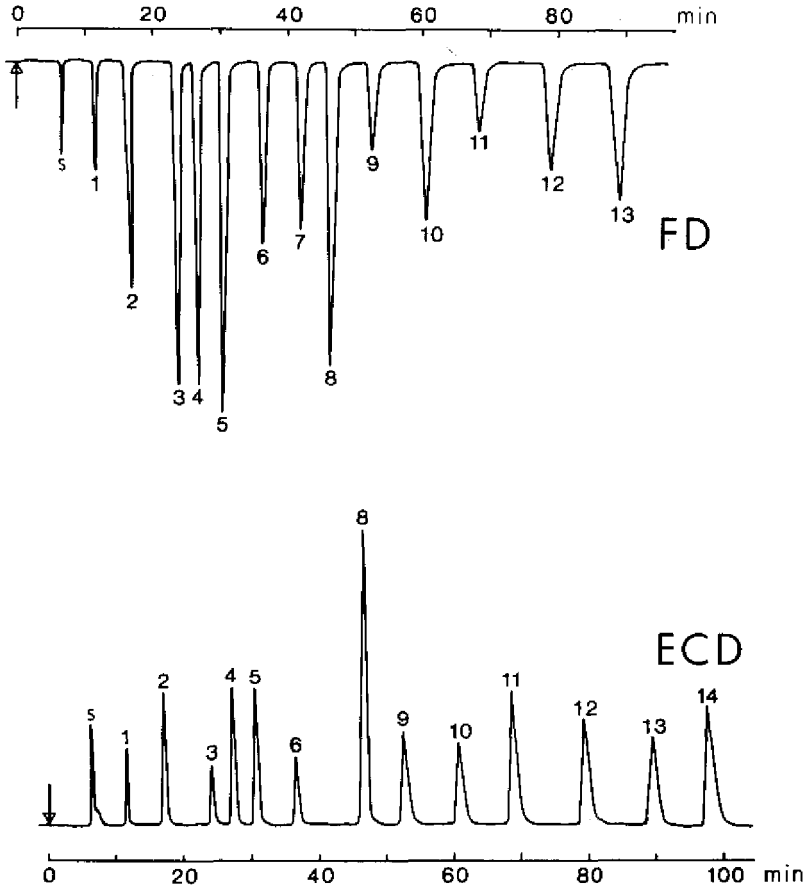

FIGURE 6 Chromatogram of a mixture of methoxylated derivatives of catecholamines, serotonin and its derivatives (group II) and internal standards (PE and IVA, not detected in ECD or in FD respectively) without the use of the column switching system: s = solvent, 1 = VMA, 2 = MOPEG, 3 = MeDOPA, 4 = NMN, 5 = 5-HTP, 6 = MN, 7 = PE, 8 = 5-Htryptophol, 9 = 5-HIAA, 10 = MOPET, 11 = HVA, 12 = 5-HT, 13 = MeDA, 14 = IVA. (FD = fluorimetric detection; ECD = electrochemical detection).

—For group II by eluting VMA, MOPEG, MeDOPA, NMN and 5-HTP through 4 columns, MN, PE, 5-Htryptophol and 5-HIAA through 3 columns (1 = 60 cm) and MOPET, HVA, 5-HT, MeDA and IVA through 2 columns (1 = 35 cm) according to the changes of column indicated by the arrows in Fig. 7. These changes were

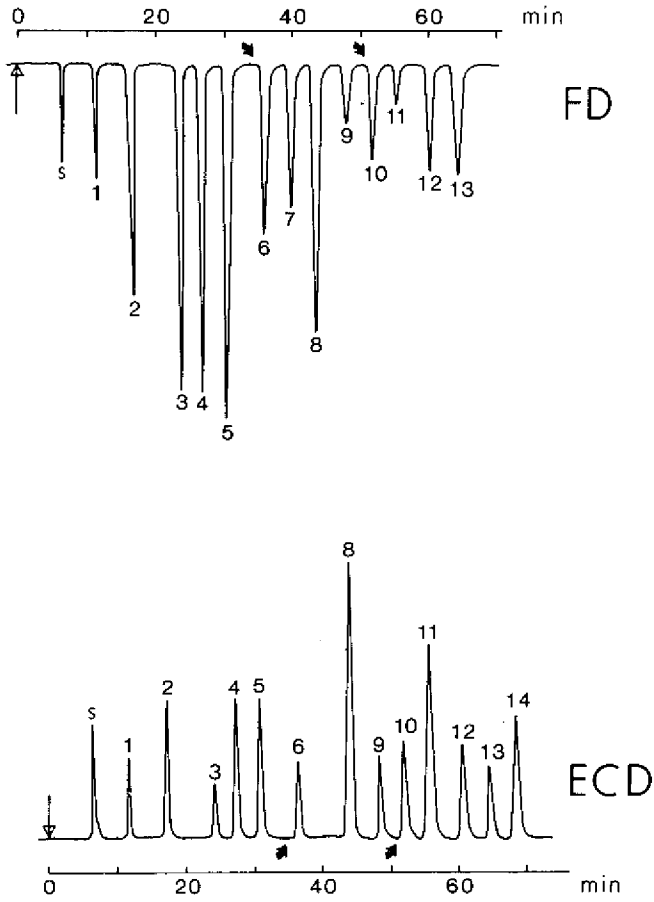

FIGURE 7 Chromatogram of a mixture of methoxylated derivatives of catecholamines, serotonin and its derivatives (group II) and internal standards (PE and IVA, not detected in ECD or in FD respectively) with the use of the column switching system: solvent (s), VMA (1), MOPEG (2), MeDOPA (3), NMN (4) and 5-HTP (5) were eluted through 4 columns (l = 85 cm), MN (6), PE (7), 5-Htryptophol (8) and 5-HIAA (9) through 3 columns (l = 60 cm), MOPET (10), HVA (11), 5-HT (12) MeDA (13) and IVA (14) through 2 columns (l = 35 cm); the arrows indicate the column switching. (FD = fluorimetric detection; ECD = electrochemical detection).

determined from the retention time of each compound and the resolution between two consecutive peaks measured after elution through each column configuration (Table II). These results showed that the resolution between 5-HIAA and MOPET ($R_s = 3.06$) and

TABLE I

Retention times of catechol derivatives and isoproterenol and resolution between two consecutive peaks for some column configurations.

Compounds	Retention times (min)				Resolution (R_s)			
	Column configurations[a]				Column configurations[a]			
	A	B	C	D	A	B	C	D
DOMA	1.4	3.9	6.5	8.6				
					NA	1.36	1.71	2.57
DOPEG	1.6	4.5	8.0	11.0				
					NA	2.22	2.38	3.18
DOPA	2.2	6.1	10.3	13.1				
					NA	1.50	1.90	2.66
NE	2.5	7.5	12.5	16.0				
					NA	2.50	3.33	4.26
E	3.4	9.9	16.2	20.9				
					NA	2.60	3.55	ND
DOPET	4.3	13.3	21.5	28.3				
					NA	2.04	2.56	ND
DOPAC	5.0	16.0	25.4	33.2				
					2.57	3.27	ND	ND
DA	7.2	20.5	33.9	44.7				
					3.11	ND	ND	ND
IP	10.7	30.6	50.2	66.1				

[a]A, B, C and D represent the length of column (Nucleosil C18, 10 μm) obtained with C1 (10 cm), C1+C2 (10 + 25 cm), C1+C2+C3 (10+25+25 cm) and C1+C2+C3+C4 (10+25+25+25 cm) respectively.
NA = Not available; ND = not determined.

between 5-HTP and MN ($R_s = 3.55$) was sufficient ($R_s > 3$) to allow the switching of the corresponding column. Contrarily to group I, due to the important number of compounds in group II and to their chromatographic behaviour, no compound was eluted only through the first column.

Consequently, the final chromatograms (Fig. 5 and 7) were obtained with the use of programs computed for Tracer–Timer as listed in Tables III and IV for groups I and II respectively.

Influence of the waiting time on the peak symmetry

Due to the important waiting time (until 30 min.) sustained by some compounds before their elution the symmetry of the corresponding peaks could be affected by a possible diffusion phenomenon.

TABLE II

Retention times of methoxylated derivatives of catecholamines, serotonin and derivatives, phenylephrine and isovanillic acid and resolution between two consecutive peaks for some column configurations.

Compounds	Retention times (min)			Resolution (R_s)		
	Column configurations[a]			Column configurations[a]		
	A	B	C	A	B	C
VMA	5.1	8.7	11.7			
				4.16	4.85	5.95
MOPEG	7.5	12.7	17.2			
				4.23	4.67	5.96
MeDOPA	11.1	18.1	24.1			
				1.14	1.60	2.22
NMN	12.2	20.0	27.1			
				1.90	2.18	2.28
5-HTP	14.1	22.9	30.6			
				2.50	3.17	ND
MN	16.5	27.7	36.5			
				1.91	2.50	ND
PE	19.3	31.5	42.2			
				1.25	2.21	ND
5-Htryptophol	20.9	35.3	46.6			
				2.01	2.40	ND
5-HIAA	23.4	40.1	52.8			
				3.06	ND	ND
MOPET	27.0	46.0	60.6			
				2.63	ND	ND
HVA	30.7	52.0	68.7			
				3.39	ND	ND
5-HT	35.7	59.2	79.2			
				2.46	ND	ND
MeDA	39.7	67.2	89.5			
				2.43	ND	ND
IVA	43.5	74.0	97.9			

[a]A, B and C represent the length of column (Nucleosil C18, 10μm) obtained with C1+C2 (10+25 cm), C1+C2 +C3 (10+25+25 cm) and C1+C2+C3+C4 (10+25+25+25 cm) respectively.
ND = Not determined.

Consequently, for each of these compounds the peak symmetry was measured following its elution according to both modes through the same length of column. The results (Table V) showed no significant differences between Mode I and Mode II elutions. This was

TABLE III

Tracer time program to eluate the catechol derivatives with isoproterenol as internal standard with the use of the column switching system.[a]

Analysis time (min)	Valves position	Event
Before start	V1, V2, V3, V4 On	Initialization.
0	V On	Injection.
9.0	V1 Off	IP is immobilized on C1.
18.5	V2 Off	DA is immobilized on C2.
19.0	V3 Off	DOPET and DOPAC are immobilized on C3 while compounds 1 to 5 are eluted through C4.
24.0	V4 Off	Stop of C4.
24.1	V3 On	Elution of DOPET and DOPAC through C3.
33.0	V3 Off	Stop of C3.
33.1	V2 On	Elution of DA through C2.
38.0	V2 Off	Stop of C2.
38.1	V1 On	Elution of IP through C1.
43.0	V1 Off	Stop of C1; end of the run.

[a]For abbreviations see text and Fig. 3.

TABLE IV

Tracer timer program to eluate the methoxylated derivatives of catecholamines, serotonin and its derivatives with phenylephrine and isovanillic acid as internal standards with the use of the column switching system.[a]

Analysis time (min)	Valves position	Event
Before start	V1, V2, V3, V4 On	Initialization.
0	V On	Injection.
15.0	V1 Off	Stop of C1.
25.5	V2 Off	MOPET, HVA, 5-HT, MeDA and IVA are immobilized on C2.
26.0	V3 Off	MN, PE, 5-Htryptophol and 5-HIAA are immobilized on C3 while compounds 1 to 5 are eluted through C4.
34.0	V4 Off	Stop of C4.
34.1	V3 On	Elution of MN, PE, 5-Htryptophol and 5-HIAA through C3.
50.5	V3 Off	Stop of C3.
50.6	V2 On	Elution of MOPET, HVA, 5-HT, MeDA and IVA through C2.
72.0	V2 Off	Stop of C2; end of the run.

[a]For abbreviations see text and Fig. 3.

TABLE V
Asymmetry factors for some compounds eluted without and with the use of the column switching system (CSS).

Compounds	Length of column (cm)	Asymmetry factors	
		Elution without CSS	Elution with CSS
DOPET	60	1.06	1.14
DOPAC	60	1.27	1.22
DA	35	1.28	1.33
IP	10	1.09	1.08
MN	60	1.50	1.48
PE	60	1.21	1.30
5-Htryptophol	60	1.02	1.08
5-HIAA	60	1.15	1.27
MOPET	35	1.05	1.03
HVA	35	1.05	1.12
5-HT	35	1.28	1.28
MeDA	35	1.05	1.09
IVA	35	1.10	1.08

confirmed in terms of peak heights which were not modified by the use of CSS comparatively to the direct elution mode.

Reproducibility

The system was used for more than 6 months. During this period the reproducibility of the observed retention times was tested. In both elution modes, only a slight decrease in the retention time of all the compounds could be observed, probably due to the progressive wear of the stationary phase.

In order to obtain a high reproducibility, several factors had to be carefully controlled:

—temperature of the mobile phase might be fixed by means of a thermostated bath (22°C ± 0.1);

—room temperature might be also maintained (air conditioning) at a value closed to that of the mobile phase;

—the effluent was day and night flowed at 0.1 ml/min during the standby periods to maintain the stabilization of columns, and the flow-rate was increased at 1.5 ml/min for 1 h before assay;

—the pH of the mobile phase was adjusted every day since this parameter markedly influenced the elution of most of the compounds studied.

Sensitivity

The sensitivity in Mode II elution was estimated as the amount of each compound which gave a peak height three-fold the background. As indicated in Table VI, the sensitivity in ECD was higher than that in FD especially for the three acidic derivatives, DOPAC, HVA and 5-HIAA which gave low FD responses. Although the detection of some compounds requires a potential higher than 0.8 V, a common 0.72 V potential was applied and appeared suitable to measure all the compounds with a good sensitivity. However the ECD sensitivity reported here was lower than that classically obtained, since in our case it was limited by the baseline variations due to the pressure shocks induced by the switching of the columns. The use of a second pulse damper would allow to reduce these

TABLE VI

Sensitivity limits in both detection modes for catecholamines, serotonin and related derivatives after elution with the use of the column switching system.

Compounds	Injected amounts (ng)	
	Fluorimetric detection	Electrochemical detection[a]
DOMA	0.50	0.07
DOPEG	0.25	0.05
DOPA	0.25	0.18
NE	0.25	0.05
E	0.33	0.09
DOPET	0.66	0.04
DOPAC	5.00	0.05
DA	0.33	0.04
VMA	1.20	0.10
MOPEG	0.50	0.08
MeDOPA	0.50	0.12
NMN	0.50	0.09
5-HTP	0.50	0.09
MN	0.80	0.05
5-Htryptophol	0.70	0.06
5-HIAA	7.00	0.15
MOPET	1.30	0.15
HVA	8.00	0.20
5-HT	1.20	0.15
MeDA	1.00	0.20

[a]With a detector sensitivity limited at 5nA full scale (see text).

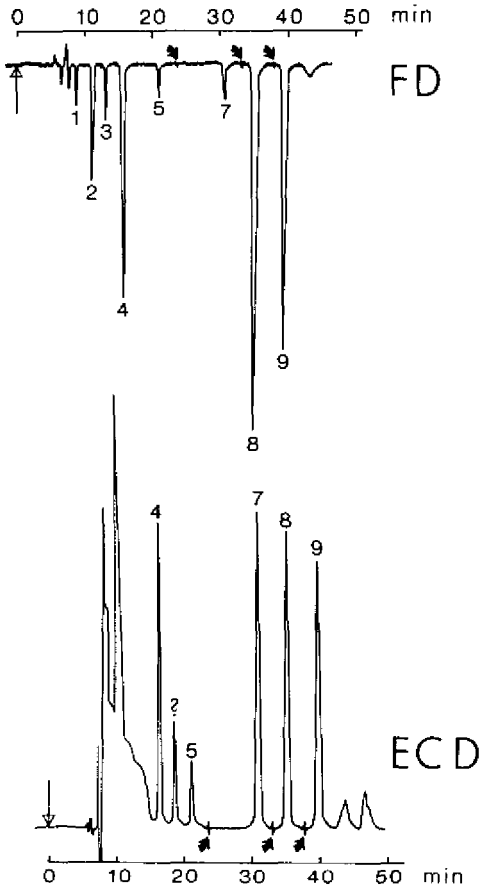

FIGURE 8 Chromatogram of a human urinary extract (catechol derivatives obtained from alumina extraction) with the use of the column switching system: solvent (s), DOMA (1), DOPEG (2), DOPA (3), NE (4) and E (5) were eluted through 4 columns (l = 85 cm), DOPAC (7) through 3 columns (l = 60 cm), DA (8) through 2 columns (l = 35 cm) and internal standard IP (9) through the first column (l = 10 cm); the arrows indicate the column switching; DOPET (compound 6 in standards) was not detectable in human urine. (FD = fluorimetric detection; ECD = electrochemical detection).

pressure shocks and to enhance the ECD sensitivity. On the other hand, the chromatographic system could be still optimized by decreasing solvent flow-rate and column length.

Linearity

The linearity was evaluated as the peak heights as well as their ratios relative to that of corresponding internal standards obtained in both elution modes as a function of the amount injected for each studied compound. In Mode I as well as in Mode II, the linearity was obtained between 5 and 500 ng for all the compounds excepted for DOPAC, HVA and IVA where it existed between 10 and 1000 ng.

Selectivity

The chromatographic selectivity for all the studied compounds has been demonstrated. The high degree of resolution ($R_s > 2$ in all cases) obtained for the separation of these compounds should be of value to free their determination of other endogenous substances expected in biological extracts. This was shown in Fig. 8 which represents a chromatogram of human urine extract obtained (group I, catechol derivatives) after alumina extraction.[29] Many other compounds were obviously present but did not interfere with those of group I. In addition, Fig. 8 shows that the important ECD solvent front did not allow to detect the most rapidly eluted compounds (DOMA, DOPEG, DOPA). On the contrary, these derivatives were easily obtained using FD. In addition, the largely retained compounds were separately and rapidly eluted through the first column by the use of CSS before next analysis. This rapid column clean-up mode allows return to the initial state.[31, 32]

Otherwise, for each compound the comparison of the ratio of the FD and ECD detector responses obtained either in biological extracts or in standards gave a valid index of the purity of corresponding peaks and therefore confirmed the selectivity of the analysis.

CONCLUSIONS

A new HPLC technique has been developed which allows the separation of 20 biogenic amines and derivatives with 3 internal standards which have to be added to the biological samples before the extraction steps.

This analysis was achieved through two runs by means of a

recently set-up automated column switching system and using on-line fluorimetric and electrochemical detections.

In such conditions the most rapidly eluted compounds were separated through the maximal length of column, while the others were eluted through a shorter predetermined length of column in order to reduce the duration of the analysis (40 and 70 min. for groups I and II respectively).

After 6 months of use the procedure has been found reproducible unless the pH and ion-pairing agent concentration of the mobile phase were carefully controlled.

From a theoretical point of view, a gradient of elution (pH, percentage of organic solvent, ionic strength of the mobile phase) might have allowed the separation of most of the studied compounds, but it would have induced a continuous baseline shift unconsistent with the high sensititivy required. In the technique described above, only slight and short lasting shifts in the baseline were observed after each change in the column length which was not opposed to the use of high sensitivity ECD.

The clear separation obtained ($R_s > 2$ in any case) for all the compounds was necessary due to the many possible interferences by endogenous compounds found in biological samples. In addition, the selectivity of the technique was enhanced by coupling on-line 2 different detectors. For each peak the relative response of these 2 detectors is a reliable index of its nature and of its purity. Furthermore, fluorimetric detection, which was less sensitive than the electrochemical one, has proven to be useful for the measurement of the rapidly eluted compounds which were masked by the solvent front in the electrochemical mode.

Finally it must be emphasized that the automated column switching system was of great value to clean up the columns and return to the baseline level before the next analysis.

In conclusion, the technique described herein offers unique possibilities for the quantification of all the known catecholamines and metabolites which can appear into urines. After improvement of the extraction procedures the use of such a method will be of great help to assess the peripheral release and inactivation of the catecholamines.

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