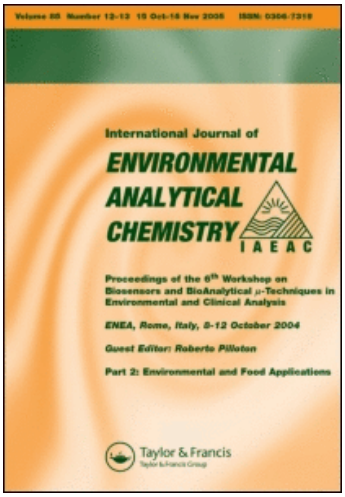

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Routine Analysis of Catecholamines and Metabolites in Urine by a Liquid Chromatographic Column Switching System†

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A reversed-phase liquid chromatographic technique including a column switching system has been adapted for the routine measurement of catecholamines and their metabolites (14 compounds) in urine. From 1 ml of urine all the compounds and the internal standards were obtained according to combined extraction procedures involving organic solvent, anionic and weakly cationic resins. Finally four extracts (catecholamines, methoxamines, acidic and neutral derivatives) had to be chromatographed throughout a wholly automated apparatus. For each run, the column switching system determined the analytical columns to be used to obtain the separation of the compounds from interferences due to other co-extracted endogenous substances, while the analysis times remained between 20 and 40 min. Such a system allowed the rapid clean-up of columns (in direct- and back-flush mode) carried out between two consecutive injections. By coupling on-line fluorimetric and electrochemical detections the specificity of the technique could be checked, since the ratio of the responses of both detectors was an index of the purity of the peaks. Finally the advanced automation of the equipment allowed weekly the evaluation of catecholamines and the whole range of their known metabolites in 36 urine samples.

KEY WORDS: Catecholamines, HPLC, urine, routine analysis.

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INTRODUCTION

The assessment of urinary catecholamines and metabolites is of considerable value in the investigation of the role of the sympathetic nervous system in various disorders including hypertension.[1]

Most of the investigators engaged in this analytical research are using advanced methods (liquid chromatography or mass fragmentography) which however are applied only to a few numbers of catecholamines and metabolites.

Since among the numerous catecholamines and metabolites some are linked by feed-back regulation processes and others derive more specifically from the central or the peripheral parts of the autonomic nervous system, an analytical method which allows us to investigate the whole metabolism of catecholamines seems to be of interest.

With this object we developed a high-performance liquid chromatographic (HPLC) column switching technique with on-line fluorimetric and electrochemical detection devoted to the measurement of free urinary catecholamines and their metabolites (14 compounds) in a single urinary sample.

The values obtained in healthy subjects maintained in controlled conditions are reported and compared to those previously published.

MATERIALS AND METHODS

Apparatus

The analytical system (Figure 1) was constituted of:

—a Model 414 high-pressure pump (Kontron, Zürich, Switzerland) with two pulse-dampers in series. An auxiliary high-pressure pump was used to perform the back-flush cleaning of columns;

—a Model MSI 660 automatic sample injector (Kontron) equipped with a 40 μl loop and provided for up to 60 sample bottles in a 2-ring disk;

—a Model MCS 670 column-switching system (Kontron) equipped with four high-pressure valves and four stainless-steel columns, the lengths of which were respectively 7 cm (C1), 10 cm (C2), 10 cm (C3), 15 cm (C4);

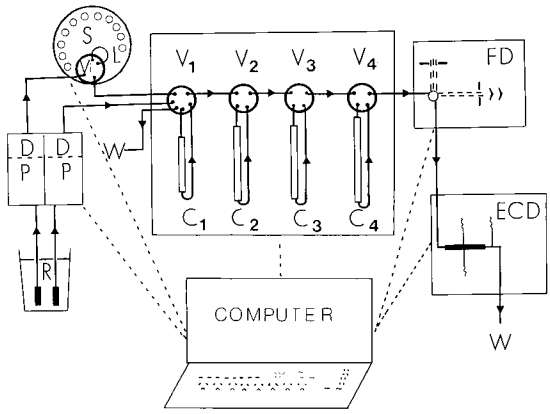


FIGURE 1 Configuration of the wholly automated HPLC system. R = solvent reservoir; P = pump; D = pulse damper; S = autosampler; L = sample loop; Vi, V1, V2, V3, V4 = six-port switching valves; C1, C2, C3, C4 = analytical columns; FD = fluorimetric detector; ECD = electrochemical detector; W = waste.

—a Model SFM 25 spectrofluorimeter (Kontron) set at 282 nm excitation wavelength and at 314 nm emission wavelength (excitation and emission slits were 10 and 15 nm respectively);

—a Model LC 4B amperometric detector (Bioanalytical Systems, West Lafayette, IN, U.S.A.) with a Model LC-17 electrochemical cell equipped with a glassy carbon electrode set at a potential of 0.8 V versus $Ag^+/AgCl$ reference electrode;

—a Model Anacomp 220 (Kontron) data system for sampling control, flow and column-switching programming, recording and integration.

The whole HPLC system was kept in an air-conditioned room (22°C ± 1).

Chemicals and reagents

Dopamine hydrochloride (DA), adrenaline (A), noradrenaline (NA), 3,4-di-hydroxymandelic acid (DOMA), 3,4-dihydroxy-phenyl-ethyleneglycol (DOPEG), 3,4-dihydroxyphenylacetic acid (DOPAC), normetanephrine hydrochloride (NMN), metanephrine hydrochloride (MN), 3-methoxy-4-hydroxyphenylethylamine hydrochloride (MT),

vanilmandelic acid (VMA), bis(3-methoxy-4-hydroxyphenylethyl-eneglycol) piperazine salt (MOPEG), homovanillic acid (HVA), isovanillic acid (IVA), 3,4-dihydroxybenzylamine (DHBA), 3-methoxy-4-hydroxybenzylamine (MBHA) were purchased from Sigma (St. Louis, MO, U.S.A.), 3-methoxy-4-hydroxyphenylethanol (MOPET) from Aldrich (St. Regis, WI, U.S.A.), 3-hydroxy-4-methoxy-benzyl alcohol (IVOH) from Janssen Chemica (Beerse, Belgium).

DHBA, MHBA, IVA, IVOH were used as internal standards (IS). All chemicals were of analytical grade and were used without further purification except methanol (Prolabo, Paris, France) and ethyl acetate (Carlo-Erba, Milano, Italy) which were distilled before use.

Stock solutions of 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 sodium metabisulfite ($Na_2S_2O_5$) and stored at 4°C in darkness.

Working solutions of catecholamines and their metabolites were diluted from their corresponding stock solutions in the same solvent to obtain various concentrations.

Working solution of a mixture of the four internal standards was diluted in the same conditions in order to add 500 ng of DHBA and MHBA, 1,000 ng of IVOH and 5,000 ng of IVA to the 1 ml urine sample.

Anion exchange resin columns were self-packed in short polyethylene columns plugged with a fritt and filled with 1 ml of a Dowex 1×4, 200–400 Mesh, Cl^- (Sigma) resin suspension in bi-distilled water. The columns were rinsed with 1 ml water before use.

Cation exchange resin columns were filled in the same way with 1 ml of a Biorex 70, 200–400 Mesh, Na^+ (Biorad, Richmond, CA, U.S.A.) resin suspension prepared as described by Jouve *et al.*[2]

The extraction columns were made of two parts: the upper one was constituted of the resin funnel and the solvent reservoir, the lower part was the collection reservoir for the waste or the elution solvent. Passages of eluents were ensured by using the centrifuge force.

Aluminium oxide was purchased from Merck (Darmstadt, F.R.G.) and prepared according to the method of Anton and Sayre.[3]

The mobile phase consisted of 93% 0.05 M potassium dihydrogen phosphate, 7% methanol, 50 mg/l disodium ethylenediaminetetra-acetate (Na_2-EDTA), 40 mg/l sodium octyl sulfate used as ion-

pairing agent. The mixture was adjusted to pH 3.3 ± 0.02 at 22°C with 15 N phosphoric acid (H_3PO_4).

The chromatographic columns were packed in the laboratory with Nucleosil C18, 10 μm (Macherey-Nägel, Düren, F.R.G.) by the upward slurry packing technique as reported by Bristow *et al.*[4] After equilibration the columns were continuously flushed (0.1 ml/min) with the mobile phase during the standby periods.

Urine collection

Urines from 15 healthy volunteers (8 women and 7 men, 22 to 34 years old) were collected during two consecutive 12-hour periods.

During the collection and the day before, the patients were maintained on a standardized diet avoiding vanillin and tyramine—rich food, coffee and fruit. The use of drugs and excessive physical activity were not allowed during the same period. Urines were kept in darkness at 4°C with 0.5 g of $Na_2S_2O_5$ and 0.5 g of Na_2EDTA were placed as preservative in polyethylene containers. At the end of the collection periods a 30 ml-sample was taken and stored at −20°C until analysis which was performed within 15 days following the collection.

Standard urine pool

In order to check the linearity of the method, six aliquots of a urine pool from healthy subjects were spiked with the mixture of internal standards and with increasing amounts of each studied compound over a range fixed between 0.5 to 10 fold the reference values reported in the literature.

Calculation

During each set of unknown samples, two aliquots of the urine pool were spiked with the highest concentration used for calibration and taken through the entire procedure.

The concentration in the unknown samples were calculated as follows:

$$\text{concentration (ng/ml)} = \frac{R \text{ sample}}{R \text{ calibrate}} \times \text{concentration in spiked urine (ng/ml)}$$

where R is the ratio of peak area of any compound to that of the internal standard, and the concentration in spiked urine is the sum of the endogenous and spiking concentrations for the compound considered.

Sample preparation (the isolation procedure is summarized in Figure 2)

The analysis of catecholamines and their metabolites required a complex extraction mode combining anionic and cationic resin adsorption with competitive elution and solvent extraction since they were compounds with chemical heterogeneity: acids, alcohols, catecholamines and methoxamines. As a consequence the extraction procedure led to four steps, the good development of which was accounted for by addition of four internal standards (IS) at the beginning of the procedure. IVA was extracted with acids, DHBA with catecholamines, MBHA with methoxamines, IVOH with alcohols, each IS being associated with the group of compounds carrying the same functional group.

Isolation of acidic metabolites DOMA, DOPAC, VMA, HVA *and* IVA (IS).

1 ml urine sample spiked with the internal standards mixture was adjusted to pH 6.5 ± 0.02 and subsequently poured onto a Dowex column. The extraction recovery was enhanced with time saving by a centrifugation procedure of the resin columns. The column effluent and a first 0.5 ml bidistilled water wash were recovered in a tube for later extraction of aminated and neutral compounds. The neutral metabolites non specifically adsorbed on the resin were eluted with a 1 ml methanol–water mixture (50:50 v/v) which was kept for the last extraction step. Finally the column was rinsed with a continuous 5 ml water wash which was discarded, then the acidic metabolites were eluted with 1.5 ml of 3 M sodium chloride (NaCl). The eluent was transferred in a glass centrifuge tube containing 100 μl 12 N HCl and 7 ml ethyl acetate and then saturated with 2 g solid NaCl. The tube was stoppered and placed on a rotating shaker (60 rpm) for 10 min. After centrifugation, back extraction was performed by mechanically shaking 6.5 ml of the organic phase with 3 ml of 0.2 M tris-HCl buffer for 10 min. After centrifugation the lower phase

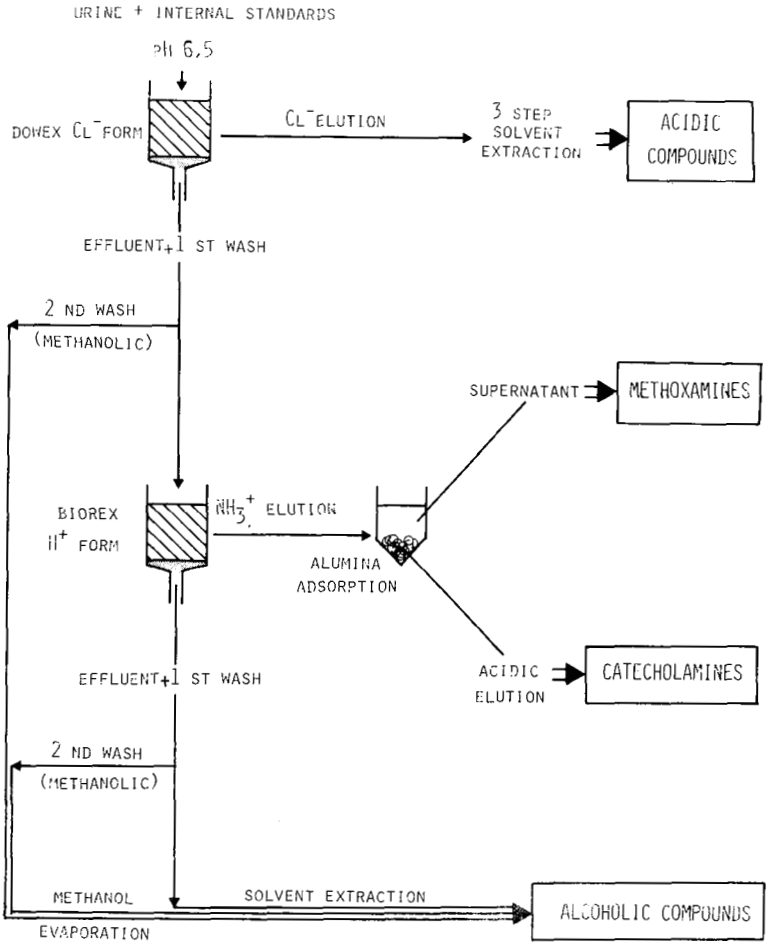


FIGURE 2 Clean-up procedure used for extraction of catecholamines and metabolites from 1 ml urine sample.

was recovered and reextracted by ethyl acetate as already described. After a brief centrifugation the organic-layer was dried over anhydrous sodium sulfate (Na_2SO_4) and then was evaporated to dryness under a stream of nitrogen in a 37°C water-bath. The residue was dissolved in 0.5 ml of mobile phase before HPLC analysis.

Isolation of catecholamines and of methoxamines NA, A, DA, DHBA (IS), *and* NMN, MN, MT, MHBA (IS).

The pooled effluents of the anionic resin column were poured onto a Biorex column. The effluent and a 0.5 ml of 0.1 M ammonium acetate pH 8.5 wash were kept for further neutral metabolites extraction. 1 ml methanol–water (50:50 v/v) was mixed to the identical Dowex one. A 5 ml twice-distilled water rinse was discarded. The compounds were eluted with 0.5 ml of 2 M ammonium acetate pH 8.5. The eluent was transferred in a 1.5 ml Eppendorf tube containing 25 mg of alumina and 0.1 ml of 7 N ammonium hydroxide. The tube was placed on an Eppendorf mixer for 10 min and centrifuged. At the end of this step, the supernatant containing methoxamines and MHBA was recovered and neutralized with 10 μl of 12 N HCl before injection in the chromatographic system. The alumina retaining catecholamines and DHBA was washed with 1 ml twice-distilled water and eluted using 0.25 ml of 0.1 M H_3PO_4. The supernatant was directly injected in the chromatograph.

Isolation of neutral compounds DOPEG, DOPET, MOPEG, MOPET *and* IVOH (IS).

The two methanol–water washes previously collected were evaporated as already described until the methanolic part was eliminated. To the residue, the pooled effluents of the Biorex column, 10 μl of 12 N HCl, 7 ml ethyl acetate and 2 g NaCl were added. The tube was stoppered and shaken for 10 min. After centrifugation the organic phase containing alcohols was dried over anhydrous Na_2SO_4, afterwards it was transferred into a conical test-tube, evaporated to dryness and reconstituted in 0.5 ml of mobile phase before injection.

Chromatographic separation

The column switching technique, as demonstrated in Cuisinaud *et al.*[5] allowed separation of catecholamines and their metabolites, serotonin and its derivatives (20 compounds) in standard solution through two runs.

According to their structural analogy, their foreseen extraction modes, and their various range of concentrations in urine, catecholamines and their metabolites extracts should be chromatographed in four runs.

The automation of the HPLC system was performed by a computer program divided for each group of compounds in three sections (Table I).

TABLE I

Typical computer program for any group of compounds studied

Section	Events
Initialization	—Establishment of flow-rate from 0.2 ml/min to 1.2 ml/min —Disconnection of C_2, C_3, C_4 —Equilibration of C_1
Analysis	—Injection —Connection and elution of columns (columns being chosen as a function of the retention degree of compounds) —Flow up to 2.0 ml/min for successive clean-up of columns —Return to analysis flow-rate —Step of the autosampler to the next sample
Termination	—Connection of C_1, C_2, C_3, C_4 —Flow down to 0.2 ml/min —Reset of the autosampler

C_1, C_2, C_3, C_4: columns 7, 10, 10, 15 cm length respectively.

The maximal length of column used for the separation of catecholamines and methoxamines was 17 cm (7 + 10 cm) while neutral and acidic compounds required 27 cm (7 + 10 + 10 cm). The analytical duration was comprised between 20 and 40 min as can be seen on urinary chromatograms (Figure 3).

RESULTS

The standard addition method was applied to a pooled urine sample to verify the linearity of the method in the expected range of concentrations to be assayed. The ratios of the peak area of any studied compound to that of the corresponding internal standard obtained in both detection modes were plotted against the concentrations added. A linear relationship was found for all the compounds as shown in Table II.

TABLE II

Regression lines obtained by standard addition of catecholamines and their metabolites to a human urine pool

Compound	Amount added (ng/ml)	Fluorimetric detection			Electrochemical detection		
		Slope	r^2	*X* intercept (ng/ml)	Slope	r^2	*X* intercept (ng/ml)
NA	0–50–100–200–500	1.0116	0.9984	89	1.0588	0.9970	104
A	0–50–100–200–500	0.8046	0.9988	37	0.6715	0.9979	30
DA	0–50–100–200–500	0.8210	0.9975	134	1.2269	0.9964	171
NMN	0–50–100–200–500	1.3790	0.9983	15	1.8150	0.9994	19
MN	0–50–100–200–500	1.3266	0.9987	17	1.5937	0.9994	19
MT	0–50–100–200–500	1.4077	0.9993	13	2.0641	0.9996	15
DOPEG	0–50–100–200–500	3.0630	0.9978	29	0.7160	0.9836	42
DOPET	0–10–20–50–100	2.6210	0.9967	2.8	0.4480	0.9942	5.1
MOPEG	0–50–100–200–500	1.2898	0.9991	39	0.9490	0.9994	46
MOPET	0–10–20–50–100	0.0751	0.9972	13	0.0866	0.9998	12
DOMA	0–50–100–200–500	1.1003	0.9973	18	0.7640	0.9905	13
DOPAC	0–100–200–500–1,000	0.1203	0.9746	2,470	0.5911	0.9810	1,330
VMA	0–1,000–2,000–5,000–10,000	1.6016	0.9917	2,760	1.5865	0.9911	3,250
HVA	0–1,000–2,000–5,000–10,000	0.3547	0.9934	2,650	1.7911	0.9917	3,040

$n=6$ for each determination.

The selectivity of both detection modes was assessed by the closed values obtained for the X-intercept which was calculated by extrapolating to zero the peak area ratio and represented the endogenous concentration.

To ensure that the procedure was quantitative and reproducible throughout a run, the within-run precision was evaluated in a urine pool and the between-run precision in a spiked urine. The highly sufficient reproducibility of the assay was confirmed by coefficients of variation lesser than 13% (Table III).

TABLE III

Within-run and between-run reproducibility of catecholamines and metabolites measurement in a human urine pool

Compound	Concentration (ng/ml)	Within-run C.V. (%) FD	ECD	Concentration added (ng/ml)	Between-run C.V. (%) FD	ECD
NA	35	2.8	2.5	500	14.0	11.0
A	14	4.3	3.9	500	3.5	3.4
DA	130	2.5	1.7	500	5.4	5.7
NMN	9	3.3	6.5	500	3.5	5.8
MN	8	5.2	4.7	500	10.0	9.7
MT	10	6.5	4.6	500	5.2	5.9
DOPEG	48	2.7	2.9	500	3.2	3.6
DOPET	—	—	—	100	6.5	8.4
MOPEG	18	2.7	1.9	500	7.4	8.4
MOPET	—	—	—	100	5.1	7.0
DOMA	36	3.3	3.0	500	9.0	10.0
DOPAC	560	3.6	2.0	1,000	6.6	13.0
VMA	900	4.8	3.9	10,000	12.7	12.0
HVA	1,250	4.5	2.7	10,000	4.3	4.7

FD: fluorimetric detection.
ECD: electrochemical detection.
C.V.: coefficient of variation.
$n=6$ for all determinations.

The results of averaged calculated recoveries listed in Table IV showed that the three chemical groups of compounds were correctly extracted.

The detection limits were determined as the amount of each compounds which gave a peak height three fold the background. The sensitivity of electrochemical detection was higher than of

TABLE IV

Recoveries of catecholamines, metabolites and internal standards in urine $n=5$ for all determinations

Compound	Recovery ± C.V. (%)
NA	72±7
A	64±7
DA	73±4
DHBA	76±4
NMN	82±5
MN	68±5
MT	72±4
MHBA	65±5
DOPEG	87±9
DOPET	23±4
MOPEG	60±7
MOPET	87±12
IVOH	68±5
DOMA	38±4
DOPAC	38±5
VMA	67±5
HVA	60±7
IVA	44±9

C.V.: coefficient of variation.

fluorimetric detection especially for acidic derivatives. A 0.8 V potential was applied to the working electrode and appeared suitable to measure all compounds with a good sensitivity.

In view of establishment of reference values for human urine the detection limits (Table V) and range of linearity were found sufficient for the determination of catecholamines and their metabolites in concentrations below and far above normal.

Typical chromatograms of urinary extracts are in Figure 3. Figure 3a shows the chromatogram of catecholamines. The separation is obtained through 17 cm length of column for NA, A, DA and DHBA (IS) and requires 20 min. The chromatogram on the left side is obtained in fluorimetric detection (FD) while the chromatogram on the right side is obtained in electrochemical detection (ECD). NA and A are clearly free of interferences in both detection modes while DA, in this case, is overlapped in ECD and hence, is measured only

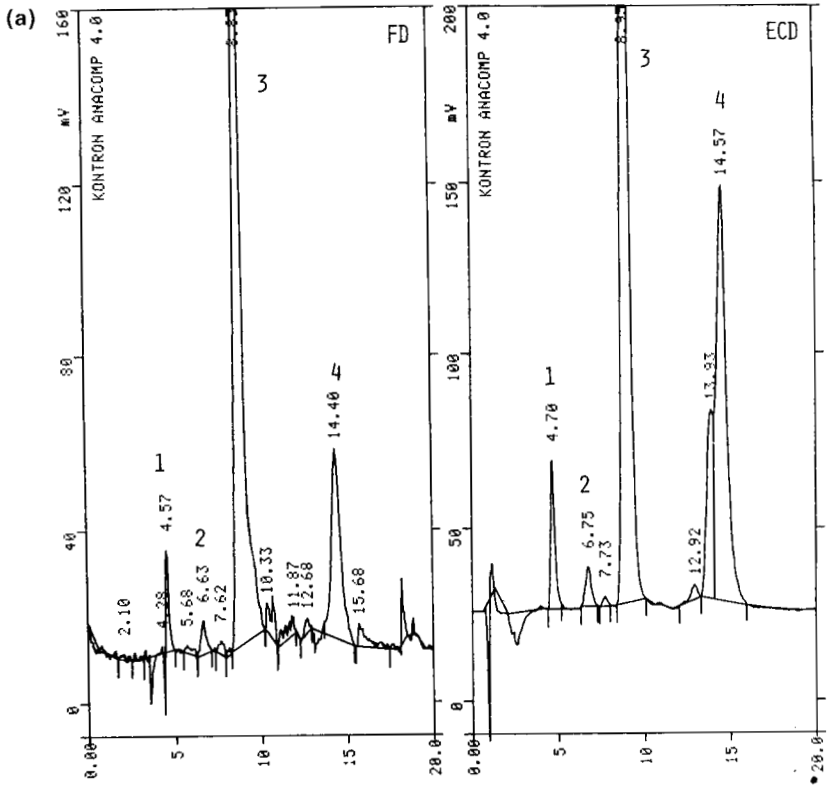
TABLE V

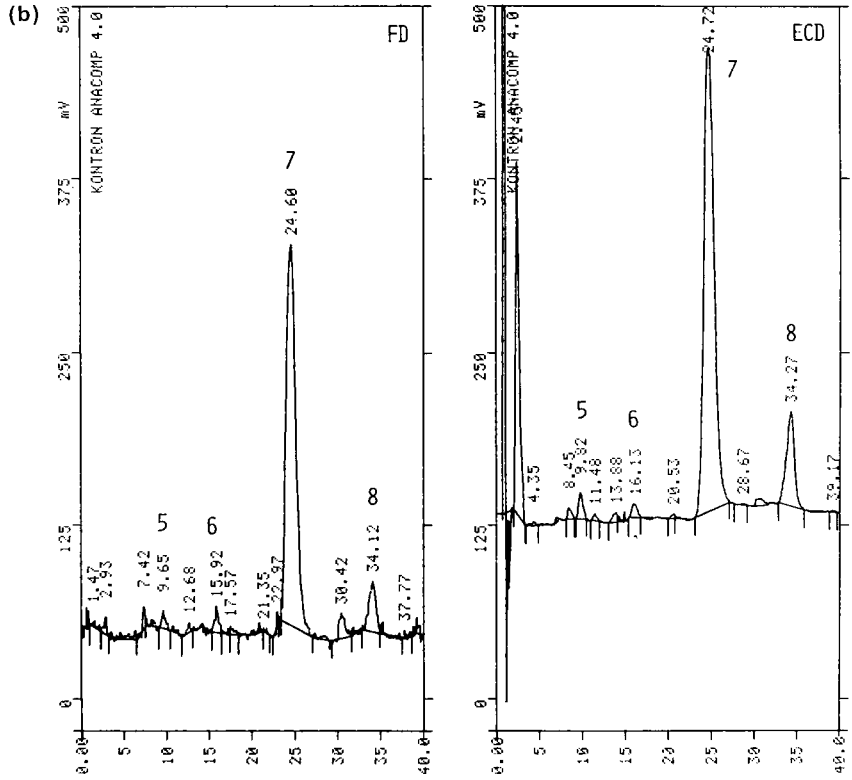
Sensitivity of catecholamines and metabolites measurement in human urine

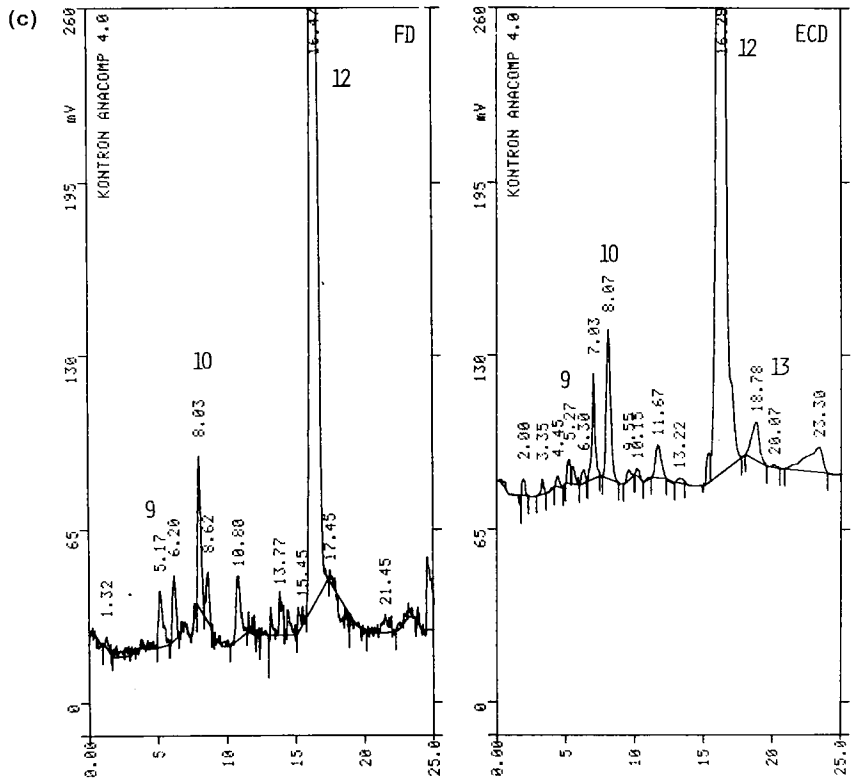
Compound	Urinary concentration (ng/ml)	
	FD	ECD
NA	5	1.5
A	5	1.5
DA	6	1
NMN	9	2
MN	9	2
MT	8	2
DOPEG	15	5
DOPET	20	2
MOPEG	6	1.5
MOPET	25	5
DOMA	25	5
DOPAC	100	15
VMA	50	5
HVA	100	10

FD = fluorimetric detection.
ECD = electrochemical detection.

in FD. Figure 3b presents a chromatogram of methoxamines. NMN, MN, MHBA (IS) are eluted through 17 cm column length, MT through 7 cm. The total analysis time is 40 min. In this case, all compounds can be easily detected in either detection mode. However, it must be noted that the concentrations usually encountered are close to the sensitivity limits of the FD. Figure 3c: DOPEG, MOPEG, DOPET are separated through 17 cm length while IVOH (IS) and MOPET are eluted through 7 cm. The analysis time is 25 min. As can be seen, an electroactive compound is eluted close to DOPEG. This unidentified peak is not detected in FD. Free urinary DOPET and in most cases MOPET cannot be measured by our method due to their too low concentration. DOMA, VMA are eluted through 27 cm column length, DOPAC through 17 cm and HVA and IVA through 7 cm (Figure 3d). DOPAC is overlapped in FD and, in addition is poorly responsive in this mode, then this compound is measured only in ECD.







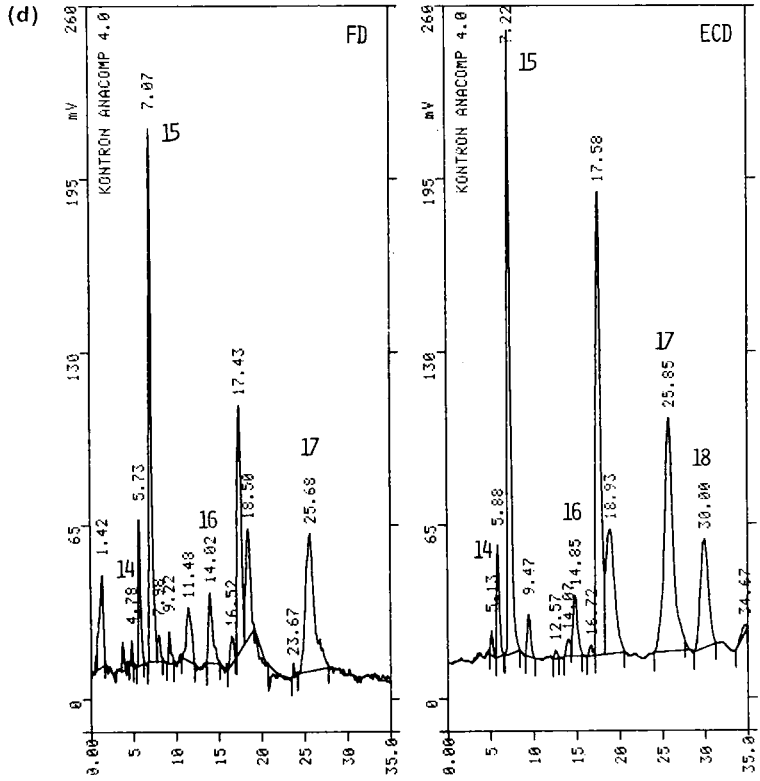


FIGURE 3 Typical chromatograms of urine extracts from healthy subjects: 3a. NA(1), A(2), IS = DHBA(3), DA(4); 3b. NMN(5), MN(6), IS = MHBA(7), MT(8); 3c. DOPEG(9), MOPEG(10), DOPET(not detected, 11), IS = IVOH(12), MOPET(13); 3d. DOMA(14), VMA(15), DOPAC(16), HVA(17), IS = IVA(18). FD = fluorimetric detection; ECD = electrochemical detection.

Urinary excretion of catecholamines and metabolites observed in 15 healthy subjects are listed in Table VI. Due to the lack of significant difference between men and women for any compound all values were pooled. Some circadian variations appear: as might be expected, there is a significant decrease in the amounts of NA and A excreted during the night ($p<0.005$) and, to a lesser extent, in the amounts of their respective methoxylated metabolites NMN ($p<0.01$) and MN ($p<0.005$). It is likely that these changes are related to the physical activity. A striking fact is that a circadian variation is observed for DOMA ($p<0.005$) and not for DOPEG. Since these compounds result both from deamination of NA after neuronal uptake, it might be suggested that they derive, at least in part, from different neuronal sources.

TABLE VI

Mean urinary excretion of free catecholamines and metabolites over 12-hour periods for 15 healthy human subjects

Compound	Amount excreted (mean ± SD) μg	
	08–20 h	20–08 h
NA	23.9 ± 12.7	14.4 ± 5.9[b]
A	6.4 ± 3.2	3.8 ± 2.2[b]
DA	130.5 ± 73.5	149.5 ± 54.5
NMN	22.2 ± 11.5	16.5 ± 11.7[a]
MN	19.2 ± 5.3	11.0 ± 4.7[b]
MT	27.1 ± 12.8	29.3 ± 15.9
DOPEG	29.3 ± 14.6	31.3 ± 10.1
DOPET	ND	ND
MOPEG	21.9 ± 4.2	18.6 ± 3.4
MOPET	ND	ND
DOMA	37.0 ± 11.9	25.3 ± 9.9[b]
DOPAC	408 ± 190	413 ± 134
VMA	1,252 ± 545	1,242 ± 620
HVA	1,424 ± 500	1,567 ± 755

ND: not detected.
SD: standard deviation.
[a] $p<0.01$.
[b] $p<0.005$ versus the 08–20 h period.

DISCUSSION

The routine analysis of free catecholamines and their metabolites reported in this paper is a specific, sensitive, linear, reproducible and relatively simple method.

The extraction procedure seems at first laborious, but as it can be seen in the literature, the analysis of several compounds with large chemical heterogeneity involves the combination of extractions with organic solvents, and the separation on cationic and anionic resins.[6] The analysis time was considerably reduced by setting up centrifugation of the resin columns. Moreover, this procedure appeared quite suitable to avoid the losses of liquid enhancing the extraction recovery and to ensure the rinses of resins.

The validity of the column switching technique in the separation of compounds of largely different polarities such as catecholamines and their metabolites was already demonstrated for standard solutions.[5] In the routine urinary analysis the column switching system permits to immobilize on the short first column endogenous co-extracted substances which are oftentimes late-eluted analytes, or, on the contrary, to eluate rapidly polar interfering compounds by progressive connection of columns at the beginning of the chromatography.

In addition such a system allows the rapid cleanup of columns (in direct or back-flush mode) carried out between two consecutive injections. The wear of the columns seemed to be longer in the direct-mode which was definitely chosen.

By using two different modes of detection a reliable index of the peak selectivity (ratio of the fluorimetric and electrochemical responses) is obtained which asserts the absence of interfering compounds.

Continuous day and night chromatography is possible owing to advanced automation of the equipment which monitors: sampling, flow and column switching programming, data acquisition and calculation. Output of full analysis then reaches 36 samples per week.

The analytical technique was applied to the establishment of reference values by young healthy subjects on two 12 hours periods. Considerable variations are reported in the urinary excretion of catecholamines and their metabolites by normal subjects. Our results

are in reasonable agreement with those previously published for catecholamines,[7-9] DOMA,[10-11] DOPAC,[10-12] VMA and HVA,[13-15] MN[16] and MT[17]. Normal values for free NMN were established only for children by Abeling *et al.*[18] Levels of free MOPEG are found far below reference values reported by most authors.[19-20] This discrepancy may be due to either the non specificity of previous methods or to the instability of MOPEG conjugates in hydrochloric medium.

As noted by Karoum *et al.*[21] for free MOPEG and free MOPET, and Howes *et al.*[22] for free DOPEG in brain, collection and storage under acidic condition may produce unconjugated compounds by hydrolysis, while the levels of other groups of assayed compounds do not change significantly. Such a deconjugation was probably the reason of the higher values reported in our previous paper for DOPEG and DOPET.[23] Regarding DOPET and MOPET our technique is not sensitive enough to detect endogenous concentrations of these compounds in their unconjugated form. However, it must be noted that other authors failed to measure these metabolites by using other techniques more sensitive such as mass fragmentography.[21]

Urine collection on Na_2EDTA and $Na_2S_2O_5$ as recommended by Moleman[24] allows after $-80°C$ conservation a one year preservation of the samples.

In conclusion, the described technique meets two essential requirements:

—first, a great versatility since it enables the analysis of any group of compounds at any moment without any change in the chromatographic conditions;

—secondly, the suitability for routine analysis since the whole HPLC technique is automated.

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