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Improvements in automated analysis of catecholamine and related metabolites in biological samples by column-switching high-performance liquid chromatography[a]

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ABSTRACT

Previously two fully automated methods based on column switching and high-performance liquid chromatography have been described, one for plasma and urinary catecholamines and the other for catecholamine urinary metabolites. Improvements in these methods, after 3 years of routine application, are now reported. The sample processing scheme was changed in order to eliminate memory effects and, in the procedure for plasma catecholamines, a pre-analytical deproteinization step was added which enhances the analytical column lifetime. The applied voltages for the electrochemical detector have been optimized, resulting in an automated method, suitable for the simultaneous determination of vanillylmandelic acid, 3,4-dihydroxyphenylacetic acid, homovanillic acid and 5-hydroxyindoleacetic acid. The sensitivity of the methods allows the detection of 2–3 ng/l of plasma catecholamines and 0.01–0.06 mg/l of urinary metabolites. Also, it is possible to switch from one method to the other in only 30 min. The normal values obtained from 200 healthy people are reported, together with a list of 57 potential interfering substances tested.

INTRODUCTION

High-performance liquid chromatography (HPLC) is usefully applied to determination of plasma and urinary catecholamines (C) and related metabolites by manual [1,2], and automated [3] methods. From the chromatographic point of view, all these analytes can be easily separated on a reversed-phase analytical column with

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electrochemical detection [1,2], but the utilization of HPLC is adversely influenced by the fact that is very time consuming to change from a kind of analysis to another. Consequently, it was considered that it would be convenient, in the context of the routine activity of a clinical chemistry laboratory, not only to automate the determination of catecholamines, but also to effect a rationalization of the sequence of various applied methods.

We could optimize the analytical process by combining three steps, sample clean-up, chromatographic separation and detection, utilizing one only instrument, specially designed for this application. For this purpose we developed a fully automated analyser, that was first applied to the determination of C [4,5].

The apparatus was constructed by connecting in series a liquid handling station, a solid-phase autosampler operating with column switching, a highly reliable HPLC pump and an electrochemical detector. Being a versatile system, it could be used also for analysing C metabolites [6] and 5-hydroxytryptamine (5-HT) [7].

The experience gained in utilizing this system for over 3 years of routine analysis and research allowed us to implement some optimizations, which are reported in this paper.

EXPERIMENTAL

Chemicals and reagents

Venous blood was drawn in tubes containing 50 μl of 0.01 *M* tripotassium ethylenediaminetetraacetate. The protein precipitating solution was 0.8 *M* 5-sulphosalicylic acid dihydrate. The urine samples were preserved with 0.1 *M* hydrochloric acid. They can be kept for up to 1 week at 4°C and for 3 months at −30°C.

The extraction cartridges, containing octadecyl-bonded silica (AASP cassette C_{18}, for C extraction) or quaternary amine-bonded silica (AASP cassette SAX, for metabolite extraction), and the packing material for the 150 mm × 4.6 mm I.D. saturation column, (customer-packed), containing Sepralite reversed-phase C_{18} (40–60 μm), were obtained from Analytichem International (Harbor City, CA, U.S.A.) and the analytical column, octadecyl C_{18}, 3 μm (50 mm × 4.6 mm I.D.), from Baker (Deventer, The Netherlands).

Instrumentation

A Model 4233R refrigerated centrifuge was supplied by ALC (Milan, Italy) and a Microfuge 12 microcentrifuge by Beckman (Fullerton, CA, U.S.A.).

The HPLC instrumentation, employing a four-solvent HPLC pump, and the sample processor were the same as previously reported [6].

Preparation of standards

To obtain standard solutions, an acidified normal urine sample was fortified with norepinephrine (NE), epinephrine (E) and dopamine (D) to give concentrations of 0, 5, 25, 100 and 500 μg/l (NE and E) and 0, 25, 100, 400 and 2000 μg/l (D). Standard solutions of metabolites were as before [6]. The calibration standards obtained can be stored at −30°C for 3 months.

Stock solutions of the internal standard (I.S.) dihydroxybenzylamine (DHBA) (15 mg/l for urinary C; 100 μg/l for plasma C) and NE, E and D (100, 50 and 100 μg/l,

respectively, for plasma C) were prepared in 0.1 *M* perchloric acid and were kept at 4°C. A stock solution of the metabolites internal standard 3-hydroxy-4-methoxymandelic acid (iso-VMA, 500 mg/l) was prepared in 0.1 *M* hydrochloric acid and kept at 4°C.

To obtain plasma C standard solutions, a catecholamine-free plasma was fortified with NE, D and E to give concentrations of 0, 50, 200, 500 and 2500 ng/l (NE, D) and 0, 25, 100, 250 and 1250 ng/l (E). These standards were prepared fresh daily.

HPLC mobile phases

The four HPLC mobile phases, A (for plasma and urinary C determination), B and C (for urinary metabolites determination) and D (for washing the HPLC system when changing from C to C metabolites determination), were prepared as follows:

(A) methanol–acetonitrile–50 m*M* NaH_2PO_4 (pH 2.8, measured as the pH of the phosphate buffer) mixed in the ratio 15:8:77 and with 200 mg/l of sodium dodecyl sulphate (SDS) added;

(B) 50 m*M* NaH_2PO_4, (pH 2.8);

(C) acetonitrile–50 m*M* NaH_2PO_4 (pH 2.8, measured as the pH of the phosphate buffer) mixed in the ratio 1:3;

(D) methanol–water (3:2).

Buffers for sample processing

The compositions of the buffers and solvents employed in the sample processing were as follows:

(i) buffer containing the complexing agent for C analysis: 2.0 *M* NH_4Cl–NH_3 (pH 8.5) buffer containing 0.2% (w/v) of diphenylborate–ethanolamine (DPBEA) and 0.5% (w/v) of disodium ethylenediaminetetraacetate (EDTA);

(ii) buffer (pH 8.5): 0.2 *M* NH_4Cl–NH_3 buffer (pH 8.5) containing 0.05% EDTA;

(iii) plasma C diluting buffer: mix, on the day of utilization, 0.5 ml of I.S. (DHBA, 100 μg/l) + 18 ml of buffer containing the complexing agent + 36 ml of buffer (pH 8.5);

(iv) urinary C diluting buffer: mix, on the day of utilization, 0.5 ml of I.S. (DHBA, 15 mg/l) + 50 ml of buffer containing the complexing agent;

(v) urinary metabolite diluting buffer: mix, on the day of utilization, 0.25 ml of I.S. (iso-VMA, 500 mg/l) + 50 ml of buffer (pH 8.5).

Analyser connections

The AASP solid-phase autosampler was connected with the sample processor (Fig. 1). The fully automated analysis consisted of four steps:

(1) Dilution of plasma or urine sample with a buffer. For C determination, this buffer must contain a complexing agent for derivatization of C into less polar compound suitable for reversed-phase extraction. After sample dilution, the automatic processor mixed the tube by air insufflation.

(2) Filling the 5-ml loop with all the solvents necessary for the sample clean-up. To keep the solvents separated, 25 μl of air were included at the end of every suction.

(3) Flushing the AASP cartridge with these solvents (load line in Fig. 1).

(4) Switching the ten-port valve to inject the sample into the HPLC column (injection line in Fig. 1).

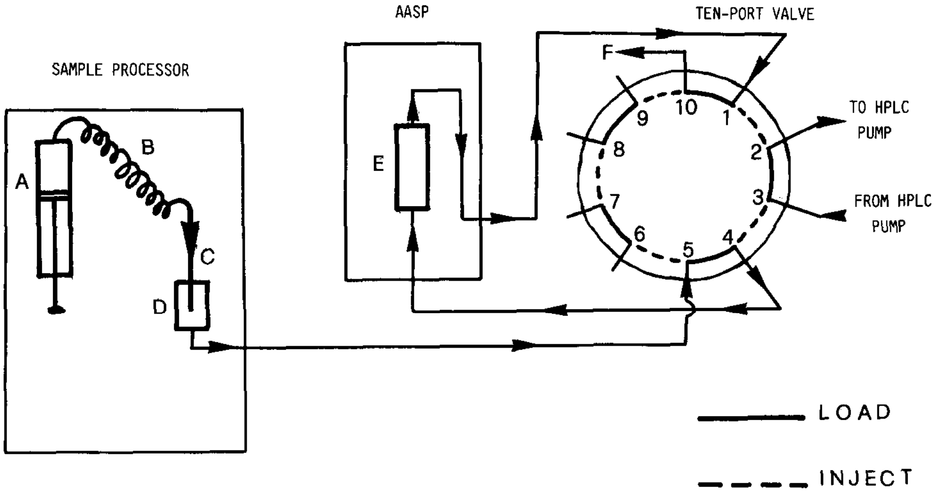

Fig. 1. Sample processing diagram. A = Syringe pump; B = 5-ml loop; C = needle of the autosampler; D = injection port; E = AASP extraction cartridge; F = purge solvent drain.

The first step can be applied to the whole batch of samples, before starting to apply steps 2, 3 and 4 to the first sample. Steps 2, 3 and 4 must be applied consecutively, completing the first sample clean-up and injection before starting with the next sample.

The diluter has a switching valve, positioned on the top of the syringe pump, which allows the syringe to suck the solution employed for washing the loop (Fig. 2,

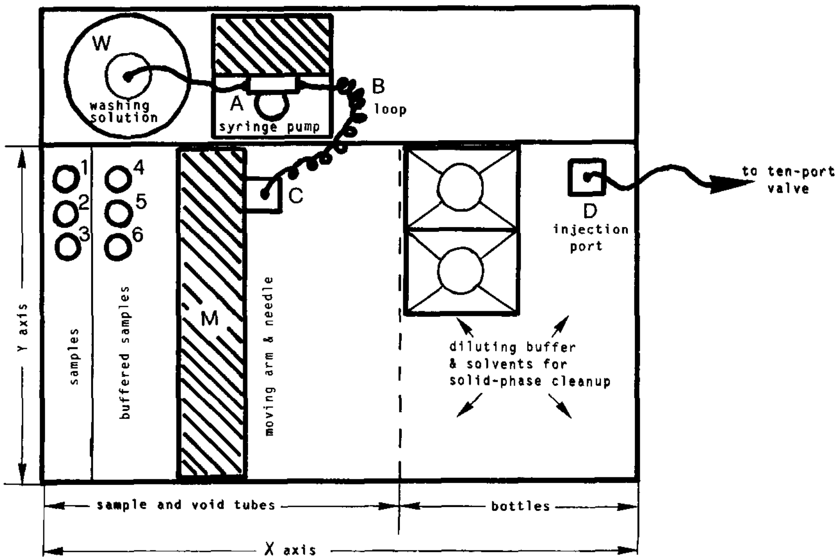

Fig. 2. Sample processor. The arm (M) moves the needle (C) along the axes X and Y on a tray containing the samples (up to 90), the buffers and the solvents (up to 8) necessary for the column switching clean-up (for details, see the text).

position W). In these applications, the loop washing step must occur just at the end of each analysis, five times, utilizing 5 ml of water.

Plasma C determination

For plasma C determination, 5 ml of venous blood were drawn and immediately centrifuged at 5500 *g* for 10 min at 4°C, then plasma samples were separated and kept at −30°C. On the day assigned for analysis, the samples were thawed at room temperature and deproteinized as follows: 0.5 ml of plasma were transferred to a centrifuge microtube and 50 μl of protein precipitating solution were added with gentle mixing. After refrigeration at 4°C for 15 min, the microtubes were centrifuged at 11 000 *g* for 10 min. The supernatant liquid was transferred to a plastic tube and positioned on the sample processor.

The details of the automated clean-up and HPLC analysis are as follows:

Step 1: sample, deproteinized plasma; dilution, 250 μl of sample + 750 μl of plasma C diluting buffer; apply step 1 to the whole batch of samples before starting to apply step 2;

Step 2: the syringe pump sucks, in sequence, 1.25 ml of water, 0.5 ml of methanol–buffer (pH 8.5) (1:4), 1 ml of buffer (pH 8.5), 0.8 ml of diluted sample, 0.5 ml of buffer (pH 8.5), 0.5 ml of methanol;

Step 3: the syringe pumps these solutions in reverse order through the cartridge (AASP cassette C_{18});

Step 4: valve reset, 0.1 min.

The HPLC mobile phase was A with isocratic elution at a flow-rate of 2 ml/min. The detector settings were as follows: conditioning cell, +0.45 V; high-sensitivity cell, detector 1 +0.10 V, detector 2 −0.35 V; gain, 100 × 10; response time, 4 s. The signal monitored (detector 2) was 10 mV; recorder, 10 mV; chart speed, 10 mm/min; and turnover time, 11 min.

Urinary C determination

Step 1: sample, acidified urine; dilution, 0.15 ml of sample +0.3 ml of urinary C diluting buffer;

Step 2: the syringe pump sucks, in sequence, 1.25 ml of water, 0.5 ml of methanol–buffer (pH 8.5) (1:4), 1 ml of buffer (pH 8.5), 0.15 ml of diluted sample, 0.5 ml of buffer (pH 8.5), 0.5 ml of methanol;

Step 3: the syringe pumps these solutions in reverse order through the cartridge (AASP cassette C_{18});

Step 4: valve reset, 0.1 min.

The HPLC mobile phase was A with isocratic elution at a flow-rate of 2 ml/min. The detector settings were as follows: conditioning cell, +0.45 V; high-sensitivity cell, detector 1 +0.10 V, detector 2 −0.35 V; gain, 10 × 5; response time, 4 s. The signal monitored (detector 2) was 10 mV; recorder, 10 mV; chart speed, 10 mm/min; and turnover, 11 min.

Urinary metabolite analysis

To switch the analyser from C to C metabolite analysis, it was necessary to wash the HPLC system with mobile phase D at a flow-rate of 1.5 ml/min for 15 min, to remove SDS from the saturation and analytical columns.

The details of the automated clean-up and HPLC analysis are as follows:

Step 1: sample, acidified urine; dilution, 50 μl of sample + 0.5 ml of urinary metabolite diluting buffer;

Step 2: the syringe pump sucks, in sequence, 0.8 ml of water, 25 μl of 0.3 *M* H_3PO_4, 0.5 ml of methanol–water (1:1), 1.0 ml of water, 50 μl of diluted sample, 0.5 ml of water, 0.5 ml of methanol;

Step 3: the syringe pumps these solutions in reverse order through the cartridge (AASP cassette SAX);

Step 4: valve reset, 0.1 min.

The HPLC mobile phase was a binary gradient between B and C with the following programme: from 2% to 10% C in 0.1 min; from 10% to 30% C in 0.9 min; from 30% to 60% C in 2.0 min; from 60% to 80% C in 2.0 min; from 80% to 100% C in 0.5 min; 100% C for 0.5 min; from 100% to 2% C in 1 min. The flow-rate was 2 ml/min. The detector settings were as follows: conditioning cell, −0.10 V; high-sensitivity cell, detector 1 +0.10 V, detector 2 +0.40 V; gain, 1 × 10; response time, 4 s. The signal monitored (detector 2) was 10 mV; recorder, 10 mV; chart speed, 10 mm/min; and turnover time, 11 min.

RESULTS AND DISCUSSION

Plasma and urinary C

For C detection the redox mode was adopted [5]. In Fig. 3, the chromatographic profile obtained by analysing urine from a healthy person is shown, and it can be seen that the analytical column, even if it is only 50 mm long, can separate the various analytes well.

In comparison with the previous model, we have changed the flow scheme. In the original set-up, the ten-port valve allowed the possibility of fitting a loop for manual injection. Problems occurred because if the treated sample contained substances eluted from the cartridge in a time range 0.1–0.2 min, the loop caused a memory effect by trapping a ghost peak, which was detected in the next sample profile, as the loop was not flushed during the sample loading step.

Fig. 4 shows a chromatographic profile obtained for plasma from a healthy person. In the procedure for the determination of plasma catecholamines, we have added a pre-analytical deproteinization step; in fact, if deproteinization is not effected one obtains an identical profile, but the analytical column lifetime is reduced and, after 190–200 analyses, the separation efficiency decreases and the back-pressure increases substantially.

On changing the column frits the back-pressure did not decrease, confirming that the problem was due to the deterioration of the stationary phase. Addition of EDTA to the mobile phase did not solve the problem, and the effect on the detector was a considerable increase in the background current and the displacement of C voltammograms, which obliged us to apply a higher voltage.

Considering that urinary C analysis did not experience such a problem, it appears clear that the effect on the analytical column was due to plasma proteins. We added 0.1% trifluoroacetic acid to the mobile phase, but the situation became worse.

We then tried to check the effect of metal parts of the apparatus: the AASP cassette cartridge frits are made of titanium, which is compatible with proteins; conse-

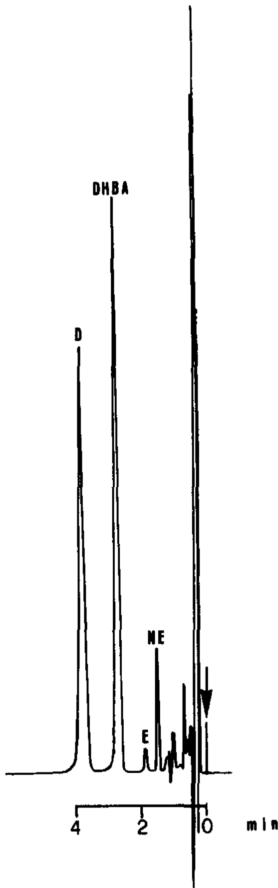

Fig. 3. Chromatogram of a urine containing NE 54 μg/l, E 10 μg/l and D 305 μg/l. Column, octadecyl C_{18}, 3 μm (50 × 4.6 mm I.D.); mobile phase, methanol–acetonitrile–50 m*M* sodium phosphate buffer (pH 2.8) (15:8:77), containing 200 mg/l of sodium dodecyl sulphate; flow-rate, 2 ml/min; detector conditioning cell, +0.45 V; detector 1, +0.10 V; detector 2, −0.35 V; gain, 10 × 5; response time, 4 s.

quently, we replaced all the tubes, including the internal ones to the AASP and those connecting with the ten-port valve. We utilized biocompatible materials, but the problem remained. Also, plasma centrifugation at 15 000 *g* did not have the desired effect.

Only after having introduced a plasma deproteinization step was the problem overcome; the average lifetime ($n = 6$) of the analytical column was optimized and no reduction in efficiency or pressure increases occurred after 800 analyses.

A further change to the original procedure was the dilution of plasma samples during step 1, the dilution rate being raised from 2:1 to 1:3. This dilution is very important, because it greatly improves the precision of the method with the most difficult samples, taken from patients affected by various and complicated pathologies.

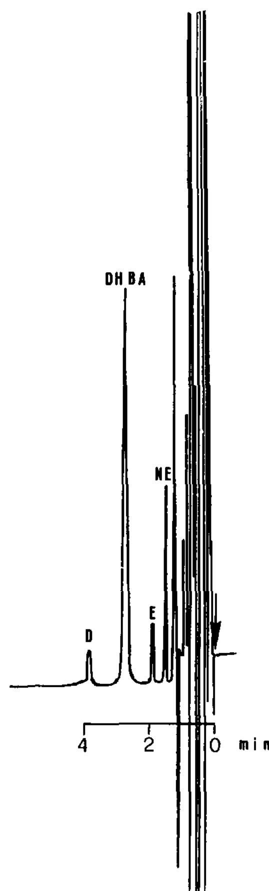

Fig. 4. Chromatogram of a plasma containing NE 82 ng/l, E 22 ng/l and D 21 ng/l. Conditions as in Fig. 3 except gain, 100 × 10.

The retention times for NE, E, DHBA and D were 1.50, 1.87, 2.70 and 3.77 min, respectively, and were not influenced either by column ageing or replacement.

The detector 2 background current (detection cell) is very low, -0.018 ± 0.002 μA, and gives a steady baseline, also at high sensitivity values. Hence it is possible to detect plasma concentrations of NE, E and D of 2, 3 and 3 ng/l, respectively. The recovery and precision of the method are unchanged in comparison with those obtained with the original method. With clinical specimens the intra- and inter-assay relative standard deviations (R.S.D.) for NE, E and D for urinary C were 2.4 and 2.8, 3.0 and 4.5 and 1.8 and 2.6% (n = 20), respectively, and for plasma C were 3.0 and 4.2, 3.4 and 5.1 and 3.5 and 5.2% (n = 10), respectively.

As previously reported, when mixed with the complexing agent, C are stable for more than 5 h at room temperature, giving the opportunity to analyse many samples in every batch [4].

In Table I are reported the up-dated normal ranges and Table II gives a list of

TABLE I

NORMAL RANGES OF CATECHOLAMINES

Catecholamines	Compound		Concentration[c]
Urinary[a]	NE		8.5 – 61.6
		$\bar{X}$ =	31.5 ± 15.30
	E		1.0 – 22.4
		$\bar{X}$ =	7.6 ± 12.53
	D		73 – 276
		$\bar{X}$ =	186 ± 63.4
Plasma[b]	NE		43 – 240
		$\bar{X}$ =	76 ± 61.4
	E		6 – 45
		$\bar{X}$ =	14 ± 22.4
	D		8 – 41
		$\bar{X}$ =	13 ± 31.7

[a] 24-h urine collection, n = 200 (100 males, 100 females); age 20–50 years.
[b] n = 100 (50 males, 50 females)
[c] Urine, μg/g urinary creatinine; plasma, ng/l.

TABLE II

HPLC RETENTION TIMES FOR CATECHOLAMINES AND POTENTIAL INTERFERING SUBSTANCES

Substance[a]	Relative retention time	Recovery (%)
Solvent peak	0.14	
HVA	0.18	<0.01
Artefact 1	0.23	
DL-β-3,4-Dihydroxyphenylalanine	0.27	<0.01
Artefact 2	0.36	
L-DOPA	0.36	3
NE	0.52	99
E	0.68	99
α-Methyldopa	0.82	3
DHBA	1.00	99
DL-Normetanephrine	1.02	0.1
α-Methylnoradrenaline	1.14	88
DL-Metanephrine	1.36	0.01
D	1.39	99
Deoxyepinephrine	1.59	76
α-Methyldopamine	1.73	4
Isoprenaline	1.82	41
Serotonine	2.77	3
3-Methoxytyramine	3.77	0.01

[a] The following substances gave no peak at all: VMA, iso-VMA, MHPG, DOPAC, 5-HIAA, β-phenylalanine, tryptophan, phenylethanolamine, octopamine, tyramine, tryptamine, tyrosine, uric acid, 3-methyluric acid, 1,3-dimethyluric acid, 1,3,7-trimethyluric acid, hypoxanthine, xanthine, creatine, creatinine, nicotine, nicotinamide, nicotinic acid, caffeine, theobromine, theophylline, promethazine, phenobarbital, salbutamol, amitriptyline, doxepin, imipramine, diazepam, nitrazepam, haloperidol, chlorpromazine, promazine, acetyl salicylate, ibuprofen, probenecid, furosemide.

potential interfering substances tested. All analytes at concentrations of at least 100 mg/l were determined with the fully automated method and those showing a peak were also injected manually to calculate the recovery. A difference in relative retention times of 0.10 is sufficient for complete (baseline) separation.

With clinical urine samples no problem due to overlapping of interfering substances was observed, as confirmed by the analysis of the voltammograms of the peaks. Ghost peaks were very unusual.

Urinary metabolites

Fig. 5 shows the voltammograms obtained for C metabolites. The voltammograms, even though maintaining the same shape, change in potential depending on the mobile phase composition, pH, cleanness and reliability of the HPLC pump. All the analytes are oxidized at an applied potential of +0.40 V, as occurs for many interfering substances. The addition of a counter ion, such as sodium heptane sulphonate or sodium dodecyl sulphate, influences the retention time of acidic metabolites, causing their reduction and decreasing the potential necessary for oxidation, so various interfering substances can be removed [6].

However, the difference among the optimized oxidization potentials is so high that it does not allow their simultaneous detection. For this reason, we removed the counter ion from the mobile phase, and to eliminate the interfering substances we operated on the potential of detector 1 (gate). Utilizing for detector 1 a potential of +0.10 V, many interfering substances are removed and the remainder can be separated with a suitable choice of HPLC elution gradient. Under these analytical condi-

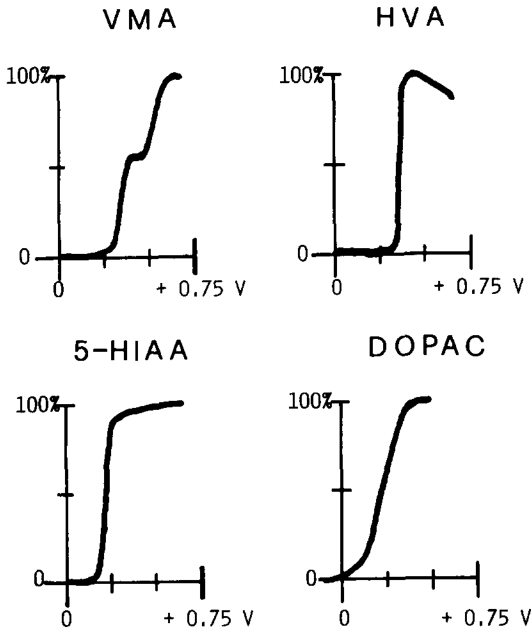

Fig. 5. Voltammograms obtained for catecholamine metabolites. Abscissa, applied voltage; ordinate, detector response, expressed as a percentage of maximum signals obtained.

tions, the conditioning cell is useless and is utilized at −0.10 V because with this potential it does not make any contribution. However, it cannot be omitted because it allows a quick transfer to the C determination stage and is useful for peak identification by recording redox-mode voltammograms.

In Fig. 6 a chromatographic profile obtained on analysing a urine sample from a healthy adult is shown. The retention times for vanillylmandelic acid (VMA), iso-VMA, 3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxyindoleacetic acid (5-HIAA) and homovanillic acid (HVA) are 1.30, 2.40, 4.10, 5.90 and 6.30 min, respectively. The recovery and precision are unchanged compared with those in the original method. With clinical specimens, the intra- and inter-assay R.S.D.s for VMA, HVA, 5-HIAA and DOPAC were 2.0 and 3.6, 1.9 and 3.0, 2.1 and 3.4 and 2.3 and 3.8% (n = 20), respectively.

The sensitivity of the method allows urinary concentrations of VMA, DOPAC, 5-HIAA and HVA of 0.06, 0.04, 0.01 and 0.01 mg/l, respectively, to be detected. In Table III the normal ranges are listed. In clinical specimens, no overlapping of interfering peaks was observed.

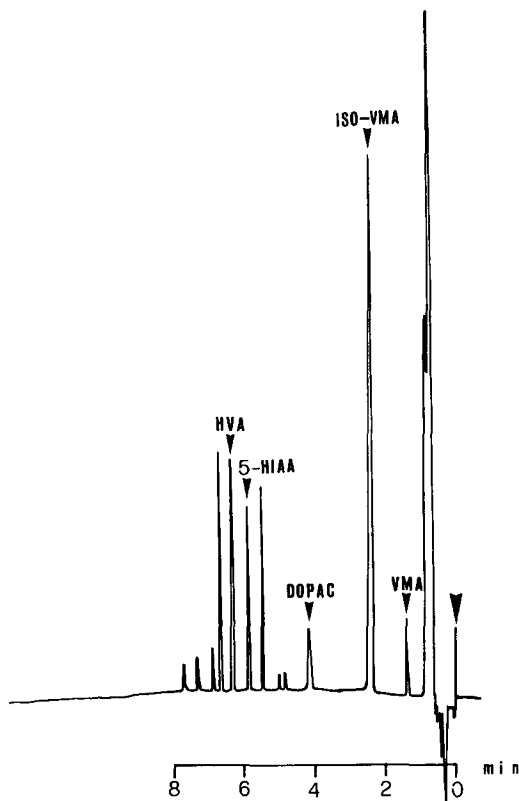

Fig. 6. Chromatogram of a urine containing VMA 2.8 mg/l, DOPAC 1.3 mg/l, 5-HIAA 2.2 mg/l and HVA 3.1 mg/l. Column, octadecyl C_{18}, 3 μm (50 × 4.6 mm I.D.); mobile phase, eluent B = 50 mM sodium phosphate buffer (pH 2.8), eluent C = acetonitrile–50 mM sodium phosphate buffer (pH 2.8) (1:3), binary gradient between B and C as described under *Urinary metabolite analysis*; flow-rate, 2 ml/min; detector conditioning cell, −0.10 V; detector 1, +0.10 V; detector 2, +0.40 V; gain, 1 × 10; response time, 4 s.

TABLE III

NORMAL RANGES OF URINARY METABOLITES

24-h urine collection; n = 200 (100 males, 100 females); age 20–50 years.

Compound		Concentration (mg/g urinary creatinine)
VMA		0.6 - 5.9
	$\bar{X}$ =	2.9 ± 2.31
DOPAC		0.2 - 3.5
	$\bar{X}$ =	1.0 ± 1.31
5-HIAA		0.5 - 6.1
	$\bar{X}$ =	2.6 ± 1.12
HVA		0.7 - 7.8
	$\bar{X}$ =	3.2 ± 2.42

CONCLUSIONS

The possibility of applying fully automated methods has many advantages. In addition to the fact that they require less manual intervention and consequently reduce random errors, only a minimum amount of sample is required for the analysis, which is of fundamental importance when the samples are drawn from very young patients (*e.g.*, aged 0–3 years). Moreover, the manual handling of unstable samples such as DOPAC (unstable at basic pH) or 5-HIAA (unstable at strongly acidic pH) can cause serious problems in their determination. The problems can be more easily overcome by applying fully automated methods, as the samples remain unaltered since they stay for less than 1 min under unstable conditions.

In the routine clinical chemistry laboratory fully automated methods can give rapid and simultaneous determinations of many substances, which allows time savings and the determination of substances present at very low concentrations and with optimum reliability of the analytical methods.

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