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FULL AUTOMATION OF CATECHOLAMINE METABOLITE DETERMINATION BY COLUMN SWITCHING AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

G. GROSSI*, A. BARGOSSI, R. BATTISTONI, A. LIPPI and G. SPROVIERI
Laboratorio Centralizzato, Policlinico S. Orsola, via Massarenti 9, 40138 Bologna (Italy)

SUMMARY

The urinary catecholamine metabolites, vanilmandelic acid, homovanillic acid, 3,4-dihydroxyphenylacetic acid and 5-hydroxyindoleacetic acid, were extracted on a silica-bonded strong-anion-exchanger cartridge (SAX) and then injected into an high-performance liquid chromatographic (HPLC) system by column switching. Chromatography was performed on a reversed-phase analytical column with electrochemical detection. Full automation was obtained by coupling two devices: a solid-phase automatic sampler and an intelligent autosampler. For each substance the recovery was >95% and the coefficient of variation was *ca.* 3%; the analysis takes 11 min. Substance instability problems are overcome, because the samples are extracted and injected in rapid succession. The normal values and correlation with manual HPLC were established for a large number of samples.

INTRODUCTION

The major metabolic end products of norepinephrine, epinephrine and dopamine, urinary 4-hydroxy-3-methoxymandelic acid (vanilmandelic acid, VMA) and 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid, HVA), respectively, are measured as an aid in the diagnosis of patients with pheocromocytoma or neuroblastoma[1]. The ratio VMA/HVA is important for the prognosis after surgical removal of the tumour[2].

Urinary VMA and HVA have been determined by liquid chromatography[3] by separating the compounds under various conditions and assaying them individually with electrochemical detectors. A method for the simultaneous analysis of VMA and HVA has been reported[4], in which urine is injected directly into a reversed-phase column and the effluent is monitored with an electrochemical detector, but the analysis time exceeded 25 min.

In previous work[5] we developed a method for urinary VMA, HVA, 3,4-dihydroxyphenylacetic acid (DOPAC, a metabolite of dopamine) and 5-hydroxyindoleacetic acid (5-HIAA, the major metabolite of serotonine) determination by reversed-phase high-performance liquid chromatography (RP-HPLC) with electrochemical detection (ED), after a solid-phase clean-up. In the clinical laboratory these

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metabolites are usually determined together with urinary and plasma catecholamines. We have also developed a fully automated catecholamine analyzer[6].

Here we present a fully automated analyzer for VMA, HVA, DOPAC and 5-HIAA, which is compatible with the catecholamine analysis. A pump metering four solvents permits all of these analyses to be performed with the same instrumentation and analytical column. It only takes a few minutes for equilibration to change from one analysis to another.

EXPERIMENTAL

Materials

The standards, VMA, HVA, DOPAC and 5-HIAA, were obtained from Sigma (St. Louis, MO, U.S.A.); sodium dodecyl sulphate was from BDH (Poole, U.K.). Extraction cartridges, containing silica-bonded quaternary amine (AASP Cassette SAX), and the packing material for the 150 mm × 4.6 mm saturation column (customer-packed), containing Sepralite reversed-phase C_{18} (40–60 μm) material were from Analytichem Int. (Harbor City, CA, U.S.A.) and the analytical column, octadecyl C_{18}, 50 mm × 4.6 mm, 3 μm, was from Baker (Deventer, The Netherlands) as was the HPLC-grade acetonitrile. All other reagents were of analytical grade. The water employed for buffers and mobile phase preparation was purified with a Milli-Q system (Millipore, Bedford, MA, U.S.A.) and the mobile phase was filtered through a 0.2-μm Durapore membrane (Millipore) and degassed. The HPLC analytical column was protected with an on-line filter (Model 7315; Rheodyne, Cotati, CA, U.S.A.).

The HPLC pump was a Model LC 21C from Brüker (Brema, F.R.G.). The Epson QX 16 computer and Epson FX 85 printer were also from Brüker. The Valco ten-port valve and the solid-phase autosampler, Model AASP, were from Varian (Walnut Creek, CA, U.S.A.) and the sampler preparator, Model 222 as well as the metering pump, Model Dilutor 401, were from Gilson (Villiers-le-Bel, France). The interface between the sample preparator and the AASP autosampler, Model Anachem, was from Gilson and the interface between the Epson computer and the Gilson sample preparator, Event Box I/O, was from Brüker.

The column effluent was monitored with a dual-electrode coulometric electrochemical ESA detector (Model Coulochem 5100 A with a 5021 conditioning cell and a 5011 high-sensitivity cell; Environmental Scienes Assoc., Bedford, MA, U.S.A.). The detector signals were recorded on a Model 56 recorder (Perkin-Elmer, Norwalk, CT, U.S.A.) and were also integrated with the Epson computer.

The optimized HPLC system was assembled by connecting in series solvent reservoirs, HPLC pump, saturation column, ten-port valve, AASP extraction cartridge, ten-port valve, filter, analytical column, conditioning cell, high-sensitivity cell, recorder and data system.

The urine samples were preserved with 0.1 *M* HCl. They can be kept up to 1 week at 4°C and for 3 months at –30°C. To obtain standard solutions, an acidified normal urine was fortified with VMA, HVA, 5-HIAA and DOPAC to give concentrations of 0.00, 6.25, 12.50, 25.00 and 50.00 mg/l. Calibration standards can be stored at –30°C for 3 months.

METHODS

In operation, the AASP solid-phase autosampler is connected with the autosampler (Fig. 1) and the extraction microcolumns (50 mg of sorbent), assembled in a cassette of ten, are substituted for the autosampler vials. The disposable extraction cartridge is pneumatically positioned on-stream with the analytical column.

The autosampler (Fig. 2) consists of a syringe pump (A) and a 5-ml loop (B), which are connected to the needle, which moves in sequence: needle washing step; 0.2 *M* NH_4Cl–NH_4OH buffer, pH 8.5, containing 0.05% of EDTA (7); void tube (4); sample (1); void tube (4); water (W); 0.3 *M* H_3PO_4; methanol–water (1:1) solution (CM); water (BF); buffered sample (4); water (BF); methanol (M); injection port (E). The autosampler dilutes 25 μl of urine in 500 μl of 0.2 *M* NH_4Cl–NH_4OH buffer pH 8.5 containing 0.05% of EDTA. Then it draws in sequence: 900 μl of water; 25 μl of 0.3 *M* H_3PO_4; 500 μl of methanol–water (1:1); 1000 μl of water; 25 μl of buffered sample; 500 μl of water; 500 μl of methanol. It pumps these solutions in reverse order through the cartridge positioned on the AASP. For the elution, the ten-port valve connects the cartridge on-stream with the analytical column for 0.2 min.

The four HPLC eluents were: (A) methanol–acetonitrile–50 m*M* NaH_2PO_4 buffer pH 2.8 (15:8:77), containing 200 mg/l of SDS (for catecholamine elution only); (B) 50 m*M* NaH_2PO_4 buffer pH 2.8, containing 200 mg/l of SDS; (C) acetonitrile–water (1:1), containing 200 mg/l of SDS; (D) methanol containing 200 mg/l of SDS. The gradient elution of the analytical column, at a flow-rate of 1 ml/min was: 100% B for 0.1 min; from 100% B to 90% B–10% C in 1.0 min; from 90% B–10% C to 80% B–20% C in 2.0 min; from 80% B–20% C to 70% B–30% C in 4.0 min; 100% B for 3.9 min.

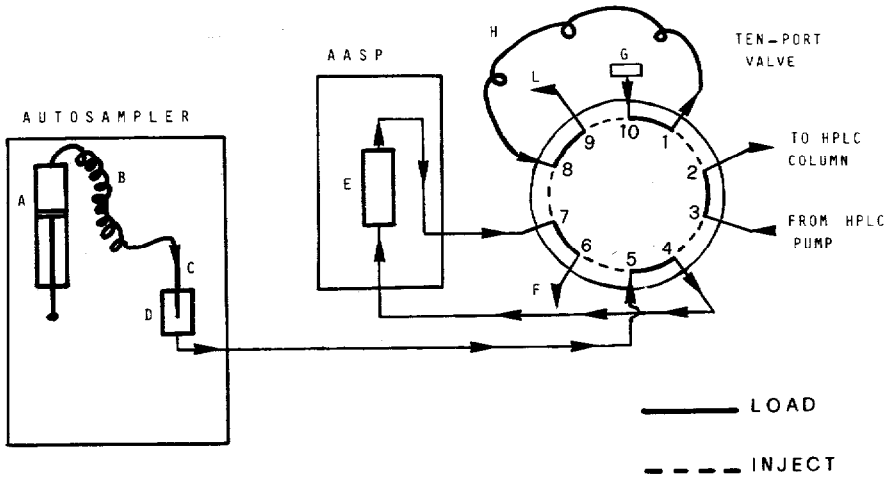

Fig. 1. Sample processing diagram. A = Syringe pump; B = 5-ml loop; C = needle of the autosampler; D = injection port; E = AASP extraction cartridge; F = purge solvent drain; G = septum for manual injection; H = manual injection loop; L = manual injection drain.

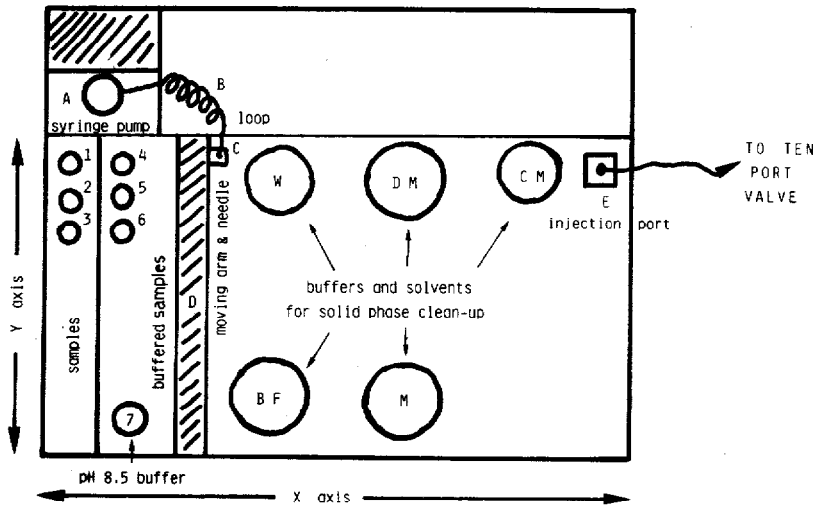

Fig. 2. Autosampler. The arm (D) moves the needle (C) along the axes *X* and *Y* on a tray containing the samples (up to 40) and the buffers necessary for the column switching clean-up. For details, see text.

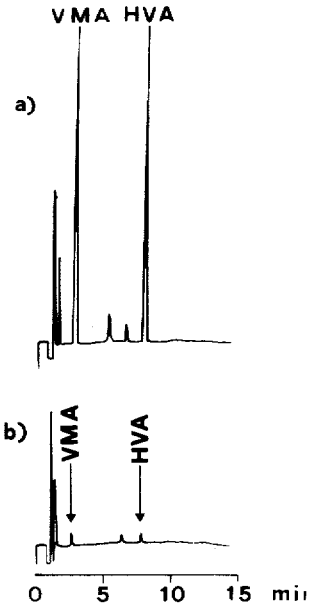

Fig. 3. Chromatogram of urine containing VMA and HVA: (a) 53.0 and 53.1 mg/l, respectively; (b) 2.2 and 1.9 mg/l respectively. Analytical conditions: column, octadecyl C_{18}, 50 mm × 4.6 mm, 3 μm; mobile phase eluent B, 50 mM NaH_2PO_4 buffer pH 2.8, containing 200 mg/l of SDS; eluent C, acetonitrile–water (1:1), containing 200 mg/l of SDS; gradient elution 0.1 min 100% B from 100% B to 90% b–10% C in 1.0 min; from 90% B–10% C to 80% B–20% C in 2.0 min; from 80% B–20% C to 70% B–30% C in 4.0 min; 3.9 min at 100% B; flow-rate 1 ml/min; detector conditioning cell, +0.00 V; det. 1, +0.00 V; det. 2, +0.35 V, gain 1 × 2, response 2.

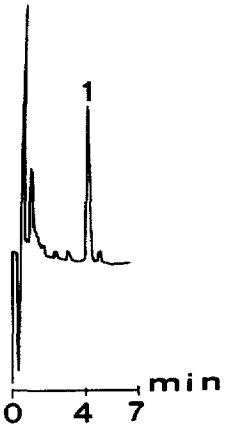

Fig. 4. Chromatogram of an urine containing 13.1 mg/l of 5-HIAA. Peak 1, 5-HIAA. Analytical conditions: column, octadecyl C_{18} 50 mm × 4.6 mm, 3 μm; mobile phase 8% acetonitrile in 50 m*M* NaH_2PO_4 buffer pH 2.8 (8:92), containing 200 mg/l of SDS; isocratic elution; flow-rate, 1 ml/min; detector, conditioning cell, –0.20 V; det. 1, –0.20 V; det. 2, +0.25 V, gain 1 × 2, response 2.

RESULTS AND DISCUSSION

Fig. 3. shows the chromatograms obtained from urine subjected to simultaneous analysis for VMA and HVA. The SDS added to the mobile phase acts by compressing the metabolite peaks and results in higher column efficiency and improved separation from artefacts.

The chromatogram of 5-HIAA is reported in Fig. 4. Isocratic elution is employed because samples for 5-HIAA usually do not have to be analyzed for VMA and HVA also.

Fig. 5 shows the chromatogram obtained for urine DOPAC determination. The retention times for VMA, DOPAC, 5-HIAA and HVA are 2.5, 4.6, 6.5 and 7.5 min, respectively. For the isocratic elution of 5-HIAA the retention time was 4.0 min. The autosampler takes 10 min to prepare a sample and the analysis time is 11 min, limited by the speed of the Gilson 222.

The minimum detectable concentration in urine is 0.1 mg/l for all the metabolites, but the sensitivity can easily be enhanced by changing the detector gain or the amount of urine extracted. The recoveries and the precision of the column-switching method are summarized in Table I. The normal ranges of metabolite concentrations were calculated for urine samples obtained from healthy adults (Table II). For 40 urine samples from healthy adults we studied the correlation between manual and automated analysis (Table III).

5-HIAA is not stable in acidic media and DOPAC is not stable in basic media; therefore manual extraction of 5-HIAA is difficult and ion-exchange manual extraction of DOPAC is impossible (for DOPAC, reversed-phase cartridges are used for manual extraction). For full automation, the sample is kept in these media for only a few seconds; no instability problems have been encountered.

By selecting eluent A as the mobile phase for isocratic elution, changing extraction cartridges to AASP cassette C_{18} and substituting Gilson buffers and solvents, the

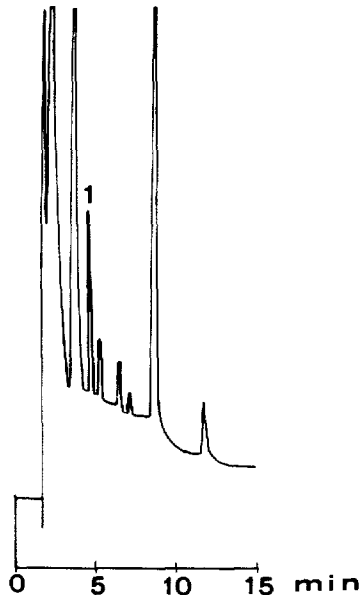

Fig. 5. Chromatogram of an urine containing 26.3 mg/l of DOPAC. Peak 1 = DOPAC. Analytical conditions are the same as in Fig. 3, but: detector, conditioning cell, +0.45 V; det. 1, –0.40 V; det. 2, +0.05 V, gain 1 × 2, response 2.

TABLE I

RECOVERIES AND PRECISION OF THE METHOD

	Fortified urine (mg/l)			*% Recovery*		
	Low	*Medium*	*High*	*Low*	*Medium*	*High*
VMA	3.1	15.1	53.0	95.1 ± 3.7	98.3 ± 2.0	99.4 ± 1.8
HVA	2,9	14.7	53.1	97.4 ± 3.1	99.6 ± 1.9	101.5 ± 1.6
5-HIAA	1.8	27.0	52.0	96.5 ± 3.6	98.2 ± 2.2	101.7 ± 2.1
DOPAC	0.9	13.5	51.0	94.3 ± 4.2	97.3 ± 2.5	99.1 ± 2.3

TABLE II

NORMAL RANGES

Compound	*Range*
Males[a]	
VMA	2.5 ± 1.25 mg/g of urinary creatinine
HVA	2.8 ± 0.95 mg/g of urinary creatinine
5-HIAA	2.8 ± 0.97 mg/day
Females[b]	
VMA	3.3 ± 1.29 mg/g of urinary creatinine
HVA	3.6 ± 1.30 mg/g of urinary creatinine
5-HIAA	2.1 ± 0.53 mg/day

[a] VMA and HVA, 7-h collection, n = 60, age 25–45 years. 5-HIAA, 24-h collection, n = 40, age 20–60 years.

[b] VMA and HVA, 7-h collection, n = 64, age 25–45 years. 5-HIAA, 24-h collection, n = 38, age 20–60 years.

TABLE III

CORRELATION OF RESULTS ON URINARY METABOLITES, ANALYZED BY TWO METHODS

X = Bond Elut extraction, manual injection; Y = column-switching extraction and injection; n = 40.

Compound	*Regression equation*	
VMA	$Y = 0.9925X + 0.1260$	$r = 0.9961$
HVA	$Y = 1.0124X - 0.0612$	$r = 0.9981$
5-HIAA	$Y = 0.9873X + 2.2561$	$r = 0.9972$
DOPAC	$Y = 0.9885X + 1.9223$	$r = 0.9987$

analyzer becomes the same as that used for catecholamines[6] and after 30 min of equilibration it is possible to start urinary or plasma catecholamine analysis (with correct detector settings).

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