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Note

Rapid and reliable estimation of urinary free catecholamines in patients with pheochromocytoma: comparison with plasma catecholamines and vanillylmandelic acid excretion

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The combination of reversed-phase high-performance liquid chromatography (HPLC) with electrochemical detection has improved both selectivity and sensitivity of catecholamine measurements [1]. The use of an ion-pairing agent in the mobile phase [2, 3] is imperative to obtain adequate retention of catecholamines by reversed-phase HPLC. The measurement of urinary catecholamines after alumina extraction alone is difficult because of chromatographic interferences [4—6]. Alternative extraction techniques [4—8] have been used to eliminate them. We now report a convenient method for the determination of urinary catecholamines using an improved extraction procedure followed by rapid separation of catecholamines by ion-pair HPLC and electrochemical detection. Application of this technique is used to compare a group of normal individuals to patients with proven pheochromocytomas.

EXPERIMENTAL

Reagents

Norepinephrine (NE) bitartrate, epinephrine (E) bitartrate, dopamine (DA) hydrochloride, 3,4-dihydroxybenzylamine (DHBA) and Amberlite CG50 (cation-exchange resin) were obtained from Sigma (St. Louis, MO, U.S.A.). Heptane sulfonic acid, octane sulfonic acid (ion-pairing reagents) and C_{18} Sep-Pak were obtained from Waters Assoc. (Milford, MA, U.S.A.). Activated alumina (AAO) was purchased from Bioanalytical Systems (West Lafayette, IN, U.S.A.). Disodium EDTA, Tris sodium phosphate and methanol (HPLC grade) were obtained from Fisher Scientific (Montreal, Canada). HPLC-grade water used in all preparations was obtained using a Millipore Milli-Q system.

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The mobile phase was 0.1 *M* sodium dihydrogen phosphate—0.1 m*M* EDTA—0.002 *M* heptane sulfonic acid, containing 3% methanol. The pH of the mobile phase was adjusted to 5.5 with sodium hydroxide. The solution was degassed before use in an ultrasound bath. Catecholamine standards were prepared at a concentration of 10 mg/ml in 0.2 *M* acetic acid and diluted before use. Tritiated norepinephrine hydrochloride, DL-[7-^{3}H]NE, was from New England Nuclear (Boston, MA, U.S.A.).

Apparatus

The liquid chromatograph used consisted a Model 6000 solvent delivery system (Waters Assoc.) with a Model U6K sample injector (Waters Assoc.) and a thin-layer electrochemical detector (Model LC4, Bioanalytical Systems). The potential was set at +0.72 V versus an Ag/AgCl reference electrode for the amines. The signals were recorded and integrated using a data module (Waters Assoc.) equipped with an internal standard program. A μBondapak C_{18} reversed-phase column (10 μm particle size, 300 × 3.9 mm, Waters Assoc.) was used.

Urine sample preparation

Urine samples (24-h) were collected in 20 ml of 5 *M* hydrochloric acid and the aliquots were frozen until use.

To a 5-ml sample of urine were added 250 ng of DHBA (internal standard). Then the sample was passed through a C_{18} Sep-Pak cartridge, which was activated by passing through 10 ml of methanol and rinsing with 10 ml of water before use.

The urine sample was passed five times through the Sep-Pak cartridge and collected. A 1.5-ml aliquot of the C_{18} eluate was then purified on Amberlite CG50 columns. The aliquot was first neutralized with 800 μl of 1 *M* Tris—20 g/l EDTA (pH 8.6). Columns were prepared in pasteur pipettes using 0.3 ml of dried powder. Columns were initially calibrated with tritiated norepinephrine to determine the elution positions and volumes of collection. The sample (2.3 ml) was applied to the column, rinsed with 0.5 ml of water and 0.5 ml of 0.2 *M* acetic acid. Catecholamines were eluted with 3.5 ml of 0.2 *M* acetic acid.

To 2 ml of the eluate were added 800 μl of Tris—EDTA pH 8.6 and 20 mg activated alumina. The eluate was then placed in an autoshaker for 20 min. The supernatant was aspirated off and the alumina washed twice with 1 ml of water. Catecholamines were then eluted with 100 μl of 0.2 *M* acetic acid and 50—100 μl were injected into the chromatograph.

Plasma sample preparation

Blood was drawn during the urine collection via an indwelling intravenous catheter into chilled tubes containing EDTA and 100 μl of a 10% solution of sodium metabisulfite. The tubes were centrifuged at 4°C within 30 min. The patient was at rest for at least 30 min in the supine position before a specimen was obtained. Plasma catecholamines were measured according to the method of Goldstein et al. [9]. After extraction with alumina the catecholamines were desorbed with 100 μl of 0.2 *M* acetic acid. The eluate (90 μl) was then injected onto the chromatographic column and separated using the system described above for urinary catecholamines. DHBA was used as the internal standard.

Calculations

The concentration of each metabolite was established from the peak areas using an internal standard program. A reference solution contained equal amounts of E, NE, DHBA and DA.

Vanillylmandelic acid (VMA)

Measurement was done in the same urine collections according to the method of Gitlow et al. [10] and expressed as mg/g of creatinine.

RESULTS AND DISCUSSION

Chromatography

The measurement of urinary catecholamines after alumina extraction is difficult because of chromatographic interferences. The pre-purification steps described above totally eliminate these interferences. This is well demonstrated in Fig. 1 which shows the HPLC profile of a control urine before (A) and after (B) pre-purification on Amberlite CG50 cation-exchange resin. Fig. 2A shows the chromatographic separation of standard NE, E, DA and DHBA and Fig. 2B the separation of a control urine using the conditions described above.

The retention times of catecholamines on a reversed-phase column depend on the addition of an ion-pairing agent. A concentration of 0.002 *M* of heptane sulfonic acid was sufficient. However, the efficiency of the column diminished over the time, and addition of octane sulfonic acid instead of heptane sulfonic acid restored the initial conditions of separation. Methanol was used as organic modifier in the mobile phase. Its concentration could be adjusted to obtain adequate resolution. In most cases, a 3% methanol concentration was sufficient. DHBA, as shown in Fig. 2, was clearly separated from E and was used as the internal standard.

Detection

The applied potential used in the present paper allows maximal sensitivity

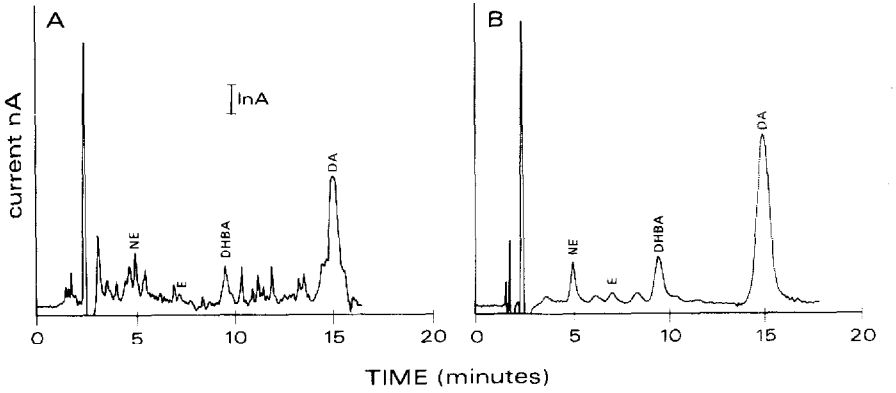

Fig. 1. Chromatogram of a control urine before (A) and after (B) pre-purification on Amberlite CG50 cation-exchange resin. Column, μBondapak C_{18}; flow-rate, 1.5 ml/min, 100 bar. Mobile phase, 0.1 *M* sodium dihydrogen phosphate—0.1 m*M* EDTA—0.002 *M* heptane sulfonic acid, containing 3% methanol; pH adjusted to 5.5 with sodium hydroxide. Detector set at 0.72 V versus an Ag/AgCl reference electrode.

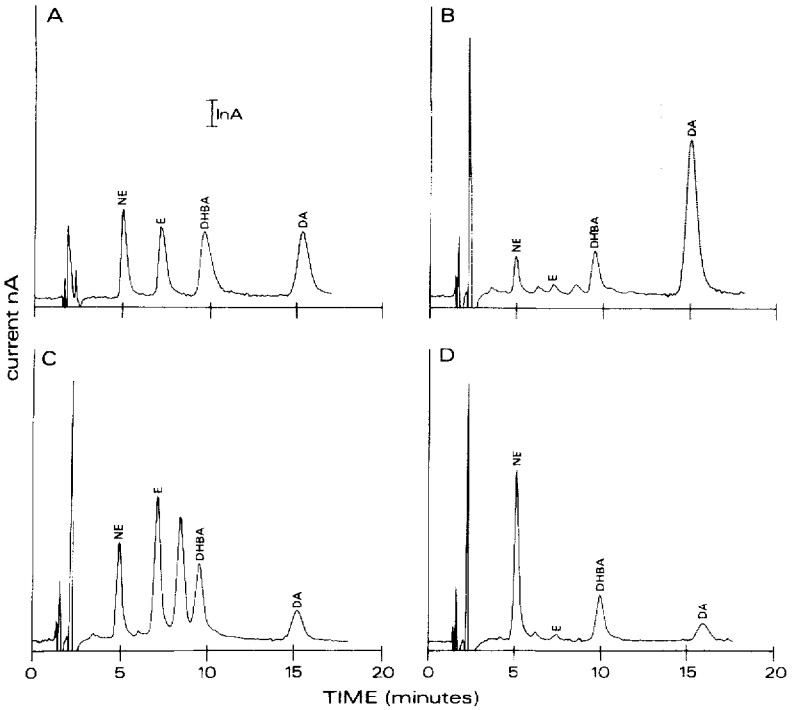

Fig. 2. Chromatograms of 5 ng of standards (A), control urine (B), urine of patient 1 (adrenal pheochromocytoma) (C) and of patient 2 (extra-adrenal pheochromocytoma) (D). Ratios of NE to E: (B) 7.6, (C) 0.58, (D) 31. Conditions are the same as in Fig. 1.

[1, 2, 9]. Setting the electrochemical detector at lower voltages offers no advantages since interferences have been removed by pre-purification.

Normetanephrine is retained on Amberlite CG50 [2] but 5-hydroxyindoleacetic acid and homovanillic acid are eliminated. Normetanephrine is not adsorbed on alumina [11].

The sensitivity is enhanced and noise decreased by polishing the glassy carbon electrode surface, replacing old electrodes, eliminating air bubbles and recycling the mobile phase. The limit of detection for standard amines was 68 pg for NE, 66 pg for E and 35 pg for DA when the electrochemical detector was set at 1 nA (signal-to-noise ratio = 2). When the detector was set at 10 nA the limit of detection was 198 pg NE, 179 pg E and 136 pg DA (signal-to-noise ratio = 2). The latter was adequate for routine analysis using 50—100 μl of urine extract. The detector was set at 1 nA for plasma samples.

Recovery

Recoveries (mean ± S.D.) after extraction and HPLC were higher for plasma catecholamines: NE, 55 ± 4%; E, 58 ± 6%; DHBA, 59 ± 5%. Individual recoveries for urinary catecholamines were as follows: NE, 40 ± 3%; E, 37 ± 6%; DHBA, 41 ± 5%. Intra- and inter-assay coefficients of variation were 5 and 7%, respectively.

TABLE I

COMPARISON OF URINARY EXCRETION OF CATECHOLAMINES, VMA EXCRETION AND PLASMA CATECHOLAMINE LEVELS IN NINE HEALTHY INDIVIDUALS AND TWO PATIENTS WITH PHEOCHROMOCYTOMA

	Urine (μg per 24h)		Plasma (pg/ml)		VMA (mg/g of creatinine)
	NE	E	NE	E	
Reference values	<60	<9.5	70—550	<110	<7
Patient 1 (adrenal pheochromocytoma)	82.5	138	182	242	9.3
Patient 2 (extra-adrenal pheochromocytoma)	1365	43.5	647	Undetectable	4.6

Urinary versus plasma catecholamines and VMA excretion

The concentration of NE, E and DA in nine normal healthy individuals were 27.2 ± 16, 3.5 ± 3 and 164.2 ± 166 μg per 24 h, respectively. The normal values for urinary free catecholamines are in agreement with previously published levels [4, 12, 13]. In Table I urinary free catecholamines are compared with plasma catecholamines and VMA excretion in patients with pheochromocytomas.

From these results, it appears that plasma and urinary catecholamines have the highest diagnostic sensitivity and urinary VMA the lowest. This is in good agreement with recent articles of Bravo and co-workers [14, 15] who have compared plasma catecholamines, urinary metanephrines and VMA. However, we find a higher increase of urinary free catecholamines than of plasma catecholamines. This may be a reflection of the activity of the tumor over a longer period of time whereas plasma catecholamines may only increase dramatically at the time of an hypertensive crisis. In addition, simultaneous measurement of E and NE may help the localization of the tumor [16]. For example, patient 1 with an adrenal tumor secretes mainly E and has a low NE-to-E ratio (Fig. 2C) whereas patient 2 with an extra-adrenal tumor secretes mainly NE and has a high NE-to-E ratio (Fig. 2D).

In conclusion, the method proposed in the present study provides a powerful tool for the diagnosis of catecholamine-producing tumors. The assay is rapid, sensitive and reproducible, and can differentiate healthy individuals from patients with pheochromocytoma easily.

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