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Note

Improved high-performance liquid chromatographic method with electrochemical detection for the determination of urinary catecholamines using a new electrochemical detector

JAYAPRAKASH B. NAIR* and MINER N. MUNK

LDC/Milton Roy, 3661 Interstate Park Road N., Riviera Beach, FL 33404 (U.S.A.)

and

JAMES D. McLEAN

Dow Chemical U.S.A., Analytical Laboratories, 1602 Building, Midland, MI 48640 (U.S.A.)

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The main pathway of tyrosine metabolism in the body is through the production of 3,4-dihydroxyphenylalanine and its direct metabolites dopamine, norepinephrine, and epinephrine. These catecholamines play a major role in the function of the body's nervous system. Epinephrine affects the rate of several metabolic processes including carbohydrate metabolism. Unusual levels of these amines and their metabolites have been associated with a variety of disease conditions such as Parkinson's disease [1], neural tumors such as pheochromocytoma [2], and hypertension [3]. These catecholamines are highly important for the identification of pheochromocytoma, for the diagnosis of carcinoid tumors, and also as biological neurotransmitters. Catecholamine excretion during stress is a subject of increasing interest [4,5]. Because of all these varied interests, there has been an active search for improved analytical methods for these compounds during the last decade.

The measurement of catecholamines in biological fluids has proved to be a difficult task because of low concentrations, susceptibility to oxidation, and complex methods of analysis. Despite these problems, the investigation of adregenic pathophysiology in man has resulted in diverse methods for the quantitation of catecholamines [6].

For high-performance liquid chromatography (HPLC), several detection techniques have been described in the literature for catecholamines [7–14]. Post-column reaction fluorimetry, native fluorimetry [12, 15], and electrochemical

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detection (ED) [12, 16–18] are some examples of the various techniques used. Detection by post-column reaction fluorimetry after converting the catecholamines to their trihydroxyindole derivatives [7, 10, 11–13] is suited for the detection of norepinephrine and epinephrine but it is not very sensitive for dopamine, as its fluorescence is weak [6, 19, 20].

The traditional fluorescence methods also suffer from several other problems. Catecholamines are present in low concentrations in urine with numerous interfering substances such as drug and natural phenolic compounds. When using fluorescent methods, the patient has to be on diet restrictions for a few days before urine is collected for analysis [21].

Detection limit and selectivity are two important factors of a catecholamine methodology. HPLC–ED provides the required detection limits for determining urinary catecholamine levels. The sensitivity of ED is comparable to that of post-column derivatization with the trihydroxyindole reaction. Since catecholamines are oxidized at a relatively low potential, selectivity is good. Urine is a complex matrix which contains many electroactive substances making pre-purification mandatory. An HPLC–ED profile of untreated urine is highly complex. Therefore the analytical method has to be selective for determining catecholamines. A specific detector such as the electrochemical detector and/or extensive sample clean-up gives selectivity. For repetitive analyses of several hundreds of samples per day the sample preparation method has to be simple and should contain the minimum number of steps, the lowest possible number of reagents and the least possible labor.

Various purification methods have been tried on urine samples for ED. Past studies have shown that a single purification step is insufficient [16, 17, 22]. Extraction with organic solvents, adsorption chromatography on aluminium oxide, ion-exchange chromatography or a combination of these procedures have been used [7–9, 16, 17]. Extensive purification after adsorption onto alumina combined with boric acid gel [16] and Sephadex [22] have been studied. These methods are generally not very specific and/or do not yield good recoveries. Among the variety of sample preparation procedures proposed in the literature for analyzing urinary catecholamines, the most common method uses ion-exchange separation and preconcentration on alumina [17]. This method has many disadvantages. When the alumina procedure is used, the mobile phase composition must be precisely adjusted (finely tuned) to prevent dihydroxyphenylglycol, uric acid, and dihydroxymandelic acid from interfering with the norepinephrine peak [23]. Moreover, this whole process takes fourteen steps and is time-consuming and labor-intensive.

Jackman [24] reported a procedure for analyzing catecholamines after elution from cation-exchange columns by monitoring their intrinsic fluorescence on excitation at 200 nm. The method is claimed to have no interference from drugs or endogenous compounds from urine. Though possibly not as sensitive as the previous assays because of sample dilution, the speed, simplicity, and freedom from significant interference make this procedure very suitable for routine use.

A similar method was independently developed in our laboratories with an improved sample preparation procedure. To the best of our knowledge, the com-

bination of this sample preparation method, in conjunction with ED, has not been reported in HPLC. In the present work, the boric acid isolation technique has been improved to avoid sample loss. Interferences in HPLC–ED profiles due to acidic components have been eliminated. Elution volume is optimized to increase catecholamine concentration giving better detectability.

This new sample preparation procedure takes only four steps. Urinary catecholamines are adsorbed onto a mini-plastic isolation column and selectively eluted with boric acid and analyzed using ion-pair reversed-phase chromatography. This method saves both time and labor. The advantages of the E.C. Monitor™ electrode over the common glassy carbon electrode are also illustrated.

EXPERIMENTAL

Reagents and chemicals

HPLC-grade acetonitrile was obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). The catecholamine standards in the bitartrate form were obtained from Sigma (St. Louis, MO, U.S.A.). 3,4-Dihydroxybenzylamine (DHBA) hydrobromide, sodium octyl sulfate, gold-label monochloroacetic acid and disodium EDTA dihydrate were purchased from Aldrich (Milwaukee, WI, U.S.A.). The urinary catecholamine mini-columns for sample preparation were obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.).

Apparatus

The HPLC–ED system for urinary catecholamines consisted of a Consta-Metric® III pump, an autosampler with Rheodyne 7126 valve, an octadecyl S5 ODS2 column (150×4.6 mm I.D., 5 μm) and the E.C. Monitor with a high-resolution working electrode (internal volume of 0.9 μl). Data were collected using a CI-10 computing integrator and printer/plotter (all components from LDC/Milton Roy, Riviera Beach, FL, U.S.A.).

Chromatographic conditions

The mobile phase was aqueous acetate buffer (pH 3) with 2% acetonitrile containing 3.0 m*M* sodium octyl sulfate as the ion-pairing reagent. The mobile phase was recycled at all times for equilibration of the system and economy of the mobile phase. The acetate buffer consisted of 0.1 *M* monochloroacetic acid–1 m*M* disodium EDTA with the pH adjusted to 3 with sodium hydroxide. The flow-rate was 1 ml/min and the injection volume 20 μl. The compounds were detected at +0.65 V vs. Ag/AgCl.

The electrochemical detector stabilized in about 20 min after initial start-up. The working electrode was sonicated in acetonitrile prior to installation in the E.C. Monitor cell.

Procedure

Urine samples were collected directly in polyethylene containers. Approximately 1 ml of 5 *M* hydrochloric acid per 100 ml of urine was added yielding a pH of 3–4 (pH paper). The samples were stored at −20°C for two weeks.

A 5-ml sample aliquot was taken for analysis and 50 μl of 0.07 m*M* DHBA-HBr

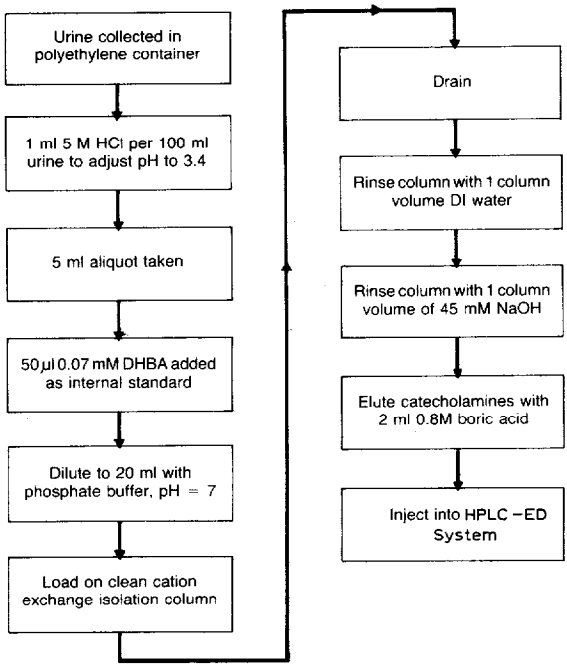

Fig. 1. Improved sample preparation procedure for urinary catecholamines.

(internal standard) were added. The sample was then diluted to 20 ml with phosphate buffer (phosphate buffer: 30 m*M* Na_2HPO_4–9 m*M* KH_2PO_4–30 m*M* Na_2EDTA; pH adjusted to 7).

The cation-exchange isolation column was drained, washed with one column volume of phosphate buffer and the prepared urine was poured into it and allowed to drain completely. The column was washed with one column volume of deionized water and then with one column volume of 45 m*M* sodium hydroxide solution. Then it was drained completely. Catecholamines are eluted with 2 ml of 0.8 *M* boric acid and injected directly into the HPLC–ED system. This procedure is illustrated in Fig. 1.

RESULTS AND DISCUSSION

When boric acid alone was used for eluting catecholamines, according to previous workers [24], many interfering peaks were seen in the HPLC–ED profile (Fig. 2). These interfering peaks were probably due to acidic components in urine which eluted near the norepinephrine peak. The interfering peaks were removed after washing the loaded ion-exchange column with one column volume of 40 m*M* sodium hydroxide solution, and the HPLC–ED profile was obtained without any interferences (Fig. 3).

It was reported that, after the catecholamines were loaded onto the ion-exchange isolation column, it was washed with 1 ml of boric acid as a clean-up step [24]. This results in loss of some catecholamines. The sodium hydroxide wash used in

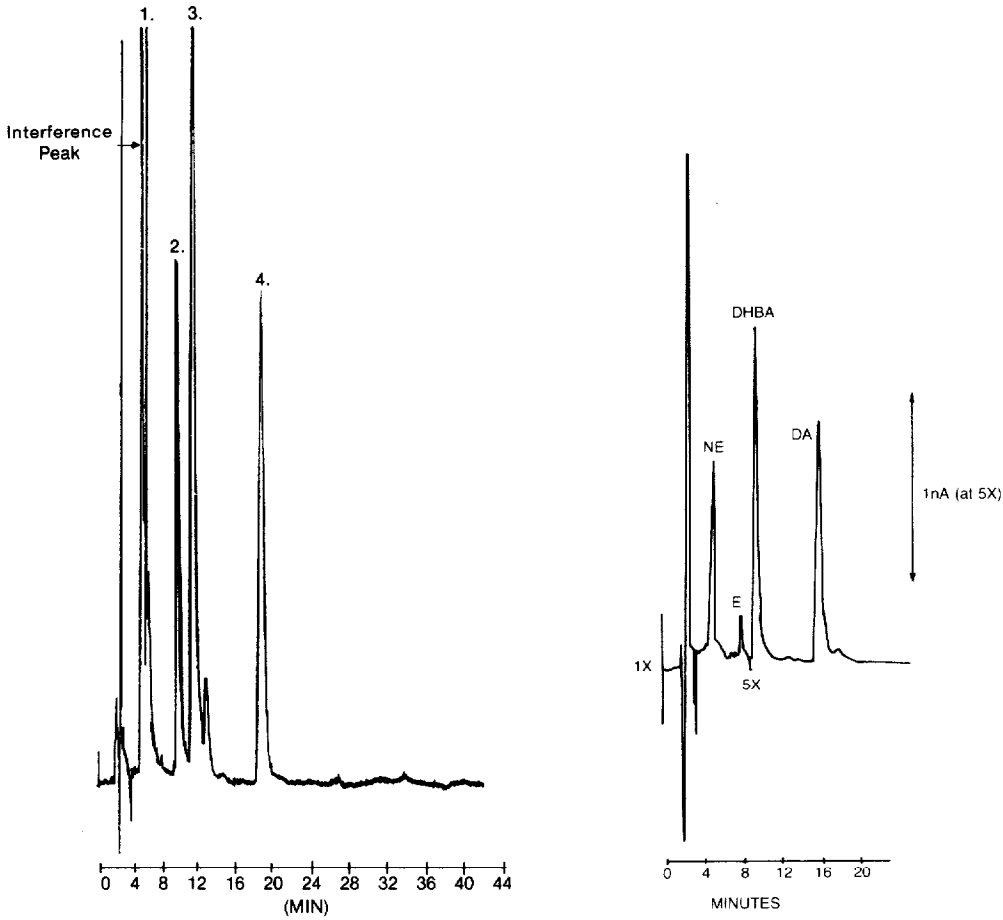

Fig. 2. Chromatogram of urinary catecholamines without the basic wash. Note the presence of interfering peaks with the catecholamine peaks. Peaks: 1 = norepinephrine; 2 = epinephrine; 3 = DHBA, internal standard; 4 = dopamine.

Fig. 3. HPLC-ED profile of urinary catecholamines after the basic wash. Note that the interfering peaks are eliminated from the chromatogram (concentrations 0.2 ng of each catecholamine). Column: octadecyl S5 ODS2; mobile phase: acetate buffer (pH 3) with 2% acetonitrile and 3.0 m*M* sodium octyl sulfate; flow-rate: 1 ml/min; injection volume: 20 μl; detector: E.C. Monitor/polyethylene carbon electrode; applied potential: $\pm$650 mV vs. Ag/AgCl. Peaks: NE = norepinephrine; E = epinephrine; DHBA = dihydroxybenzylamine, internal standard; DA = dopamine.

our study does not elute the catecholamines, but selectively removes acidic components. In this method the volume of the resin eluate is only 2 ml. Therefore the concentration of catecholamines in the eluate is high. Only 20 μl of this eluate is injected into the HPLC–ED system. The detection limits were of the order of 100 pg for norepinephrine with a signal-to-noise ratio of 3.

The method is free from interference by drugs or endogenous compounds in urine. The day-to-day coefficient of variation is less than 7.2% at a physiological level and the within-day variation is less than 4.5%.

The alternative methods of sample preparation, such as using alumina in addition to the ion-exchange column, require several additional steps, reagents, and time. Moreover alumina can absorb catecholamines, and this absorption is not always quantitative. These findings suggest that the alumina treatment procedure optimized for catecholamines [17] is not the best for extraction and preliminary purification of catecholamines in biological samples. The present method provides a fast and rapid sample preparation procedure with minimum labor and materials. There are no interferences in the chromatogram. Using this method, the recovery (mean $\pm$ S.D.) for the urinary catecholamines is $82.8 \pm 3.2\%$ at a concentration of 10 ng/ml. Standard curves using DHBA as an internal standard were linear for concentrations from 0.1 to 10 nmol/ml.

Uniqueness of the E.C. Monitor electrode

The E.C. Monitor used in this methodology has certain new and unique features. The passivation of the surface of solid electrodes is one of the most serious problems occurring in the application of electrochemical methods for routine analysis [25]. Surface passivation causes a gradual change in electrode activity and corresponding changes in the signal with time as in the case of glassy carbon electrodes. Therefore, amperometric detectors with glassy carbon working electrodes require frequent electrode surface polishing. This renewal of the glassy carbon surface is achieved by a variety of methods [26]. The most common method is to polish it with an abrasive material, such as alumina, silicon oxide, or diamond slurry.

The E.C. Monitor uses a unique, patented material for the working electrode [27, 28] to minimize surface contamination and hence electrode passivation seldom occurs. The tubular electrodes are inexpensive, disposable, and easy to install. The electrodes come ready to use and do not require prior polishing or preparation. Unlike carbon paste electrodes, the electrode is inert to eluents containing organic modifiers such as acetonitrile, methanol etc. The modified E.C. Monitor tubular electrode has a cell volume of 0.9 μl which causes minimum band dispersion and facilitates high resolution. Therefore, this detector could conceivably be used for microbore applications as well.

Tubular electrodes are simple to use and have excellent hydrodynamic properties [29]. When they are made of commonly used electrode materials, it is difficult to polish and clean their inner surfaces, because of the small hold-up volume. Therefore, tubular electrodes have not gained wide acceptance in LC amperometric detectors [29]. With this new carbon/polymer electrode, no prior preparation or frequent polishing is required – in contrast to glassy carbon. The same working electrode can be used for months without replacement. The polyethylene-based electrode material forms a smooth inner surface from the heat of drilling, thus avoiding any additional user polishing requirements.

HPLC coupled with amperometric detection is the present method of choice for catecholamines. We conclude that the combination of this specific and easy to handle short-step procedure (cation-exchange adsorption and boric acid elution) to isolate urinary free catecholamines and the high resolving power of reversed-phase HPLC coupled to the high sensitivity of the electrochemical

detector (E.C. Monitor) provides a rapid and reliable method for the determination of the three urinary free catecholamines.

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