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Note

Liquid chromatographic determination of urinary catecholamines after one-step alumina extraction

GEORGE M. ANDERSON*

Department of Laboratory Medicine and The Child Study Center, Yale University School of Medicine, P.O. Box 3333, New Haven, CT 06510 (U.S.A.)

and

TRACY A. DURKIN, JUDITH B. MORTON and DONALD J. COHEN

The Child Study Center, Yale University School of Medicine, New Haven, CT 06510 (U.S.A.)

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Over the past ten years a large number of high-performance liquid chromatographic (HPLC) method have been developed for the determination of urinary catecholamines. The determinations differ in terms of the method of sample purification, mode of chromatography, and type of detection. In general, fairly intensive sample purification was found to be necessary even when followed by highly selective analytical separations.

One group of methods [1–4] involves liquid chromatography with electrochemical detection (LC–ED) of the catecholamines after a two-step purification procedure (cation-exchange column and alumina batch extraction) and reversed-phase ion-pair chromatography. Two closely related LC–ED methods [5, 6] employ slightly different multi-step sample purifications. Four additional LC–ED methods have been reported which employ single-step sample purifications. These involve ion-pair chromatography after either ion-pair extraction [7] or in-line pre-column alumina [8, 9] extraction, or cation-exchange chromatography following batch alumina extraction [10].

Alternatively, the catecholamines have been detected fluorometrically. Initially it was found to be necessary to react the compounds so as to form fluorescent derivatives [11, 12]. More recently, two methods which measure the native fluorescence of the catecholamines have been described [13, 14]. Our previously reported native fluorescence method [13] employed a two-step sample purification step identical to that used in several of the LC–ED methods [1–4]. Here we

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report a simplified native fluorescence detection (FD) method for urinary catecholamines which requires only a single-step batch alumina purification step before reversed-phase chromatography with a dodecylsulfate-containing mobile phase.

EXPERIMENTAL

Chemicals

Norepinephrine (NE) bitartrate, epinephrine (E) bitartrate, dopamine (DA) hydrochloride, dihydroxybenzylamine (DHBA) hydrobromide, sodium dodecylsulfate (SDS), Tris base, and alumina (neutral, grade 1, acid-washed) were purchased from Sigma (St. Louis, MO, U.S.A.). Acetonitrile and methanol (distilled in glass) were purchased from American Burdick & Jackson Labs. (Muskegon, WI, U.S.A.); all other chemicals were reagent grade from local suppliers. Stock solutions of the standards (10 mg as free base in 100 ml of 0.2% ascorbic acid) were prepared every twelve months and stored in 8-ml aliquots at −70°C. Aliquots of stock were thawed and used for one month to prepare fresh daily 1 ng/μl dilutions in 1 *M* acetic acid. The mobile phase was prepared by adding 27–30% of acetonitrile to 70–73% of 0.1 *M* sodium phosphate buffer (pH 3.5) containing 1.0 g/l SDS and 25 mg/l Na_2EDTA.

Apparatus

The chromatographic system was composed of an Altex 110A pump, Rheodyne 71-25 injector, 25 cm × 0.46 cm I.D. Altex Ultrasphere C_{18} reversed-phase column (5 μm) (Raisin Instruments, Woburn, MA, U.S.A.) and a Shimadzu RP-350 flow-cell spectrophotofluorimeter (Shimadzu, Gaithersberg, MD, U.S.A.). Excitation and emission wavelenglths were 282.5 and 317.5 nm, respectively (bandpasses 20 nm). For LC–ED comparison studies, an LC-3 controller and TL-4 glassy-carbon electrode (+0.7 V vs. Ag/AgCl) were used (Bioanalytical Systems, West Lafayette, IN, U.S.A.).

Procedures

Timed, refrigerated urine specimens were collected with 1 ml of 6 *M* hydrochloric acid per hour added beforehand. After measuring the total collection volume, 15-ml portions were prepared for storage by adding 3 μg of DHBA, 100 μl of 5% metabisulfite and 100 μl of 10% Na_2EDTA. The procedure was scaled-up in order to prepare approximately 50 aliquots of pooled urine to serve as a quality-assessment sample.

The catecholamines were extracted from 2-ml portions of the thawed urine samples by adding 5 ml of distilled water, 400 μl of 5% (w/v) sodium metabisulfite, 2 ml of 3 *M* Tris buffer (pH 8.6), and 100 mg of acid-washed alumina. The pH values of the mixtures were checked and adjusted to between 8.4 and 8.7 if necessary. The capped tubes were mixed by rotation for 10 min at room temperature. The supernate was withdrawn, and the alumina washed twice with 2 ml of distilled water (pH adjusted beforehand to 6.0). After carefully removing as much of the second wash as possible, the catecholamines were eluted in 200 μl of 1 *M*

acetic acid. The alumina extracts can be analyzed directly or after long-term storage at −70°C. In either case 20–50 μl of the extracts were injected on the reversed-phase ion-pair LC–FD system described above; flow-rate, 1.5 ml/min. Standards were injected every eight samples, concentrations usually are calculated by rationing peak heights of the catecholamine and DHBA in the sample, multiplying by the standard peak-height ratio, and then by the concentration of added DHBA (e.g. (sample NE/DHBA) × (standard DHBA/NE) × 200 ng/ml).

RESULTS AND DISCUSSION

A chromatogram of free catecholamines determined in a urine sample is shown in Fig. 1. Retention times for NE, E, DHBA, and DA were 6.1, 7.1, 8.2, and 9.6 min, respectively. Fluorometric detection limits of 60–90 pg were observed (signal/peak-to-peak noise ratio of 2). The within-day coefficients of variation for the samples were 1–5% (NE, 1.2%; E, 4.8%; DA, 0.8%; $n=4$). Day-to-day coefficients of variation of 3–12% (NE, 2.7%; E, 11.9%; DA, 11.6%; $n=6$) were obtained. Recoveries of the compounds were checked by analyzing spiked samples ($n=8$). Absolute recoveries (mean ± S.D.) of NE, E, DHBA, and DA were 60.8 ± 3.3% ($n=11$), 61.8 ± 5.2% ($n=11$), 63.5 ± 77% ($n=20$), and 63.9 ± 4.0% ($n=11$), respectively, when 200 ng/ml NE, 50 ng/ml E, 200 ng/ml DHBA, and

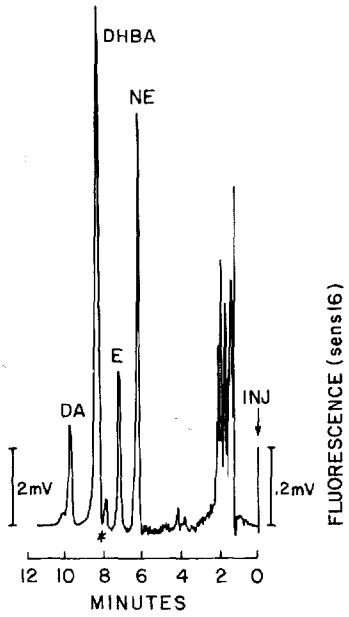

Fig. 1. Urinary free catecholamines (NE, E, DA) determined by injecting 25 μl of a 200-μl alumina extract of 2.0 ml of urine. The catecholamines were detected fluorometrically after ion-pair (SDS) chromatography (1.5 ml/min) on a 25 cm × 0.46 cm I.D. reversed-phase column. Concentrations for the sample shown are 17.2, 7.8, and 68.4 ng/ml for NE, E, and DA, respectively (200 ng/ml of the internal standard, DHBA, was added). Asterisk indicates change in recorder sensitivity from 2 to 20 mV full scale.

1000 ng/ml DA were added to urine. Consistent recoveries were observed for NE (54.8 ± 1.9%), E (55.3 ± 3.1%), DHBA (53.1 ± 2.0%), and DA (55.5 ± 1.7%) when the amount of standard added was varied over a wide range (NE, 50–400 ng/ml; E, 10–80 ng/ml; DHBA, 25–200 ng/ml; DA, 200–1600 ng/ml; $n = 4$–5).

The identities of the putative catecholamine peaks observed in urine samples were checked by several means. First, a group of samples also were determined using amperometric detection. Although the LC–ED chromatograms had significantly more extraneous peaks, the LC–FD and LC–ED methods were highly correlated. Correlations (r) for NE, E, and DA determined by LC–FD and LC–ED were 0.991, 0.991, and 0.999, respectivley ($n = 11$). Mean values (LC–FD versus LC–ED) in the group of eleven samples were 17.1 versus 15.7 ng/ml (NE), 7.00 versus 5.81 ng/ml (E), and 87,5 versus 90.3 ng/ml (DA). Second, NE, E, and DA peaks from a pooled urine sample were collected and reinjected on a 15 cm × 0.46 cm, 5-μm C_{18} Altex Ultrasphere column eluted (at a flow-rate of 1.0 ml/min) with a mobile phase consisting of 15% methanol and 85% 0.1 *M* sodium phosphate buffer (pH 4.0) containing 100 ml/l sodium octylsulfate. Single, well shaped peaks were observed after fluorometric and amperometric detection, and quantitation of the collected fractions gave results in agreement with the original analyses.

A large number of drugs were injected directly (2.5–10 μg) to test for potential interferences. None of the drugs tested interfered with the peaks of interest: imipramine, desmethylimipramine, procainamide, N-methylprocainamide, phentolamine, clonidine, yohimbine, nicotine, diazepam, propranolol, 2-hydroxydesmethylimipramine, L-DOPA, carbamazepine, caffeine, guanidine, isoproteranol, furosemide, aldomet, quinine, hydralazine, thiazide, hydrochlorothiazide, chlorthalidone, and spironolactone.

The selective and sensitivity of the method allow the catecholamines (NE, E, DA) to be determined easily at normal and below normal levels in human urine. When injecting 50 μl of a 200-μl alumina extract of 2.0 ml of urine, the catecholamines in approximately 0.3 ml of urine are detected (assuming a 60% recovery). Due to the selectivity of the SDS ion-pair chromatography, the absolute instrumental detection limits of 60–90 pg can be approached with urine samples, resulting in detection limits of approximately 0.2 ng/ml. These detection limits are well below the usual normal levels seen for NE, E, and DA (25, 5, and 200 ng/ml, respectively). The compounds also have been determined in urine after hydrolysis (30 min at 90–100 °C, pH 1.5 adjusted with hydrochloric acid); the resulting chromatograms were free of interfering peaks.

In summary, we believe the method to be one of the simplest, most rapid, more sensitive methods available for the determination of human urinary catecholamines. While measurements of urinary catecholamines have acknowledged utility in diagnosis of certain clinical conditions, we would like to suggest that their potential in assessing sympathetic nervous system (SNS) and adrenal functioning in neuropsychiatric disorders has not been appreciated fully. While it is probably necessary to measure NE and E in plasma in order to detect very rapid and transient changes (< 1 h) in their secretion, more long-term changes in, or dif-

ferences in, SNS and adrenal functioning probably can be better studied by examining urinary excretion rates of the catecholamines.

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