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Note

Modified sample preparation for high-performance liquid chromatographic—electrochemical assay of urinary catecholamines

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High-performance liquid chromatography with electrochemical detection (HPLC—ElCD) has gained wide acceptance as a method for measuring catecholamine concentrations in physiological fluids. Advantages of the HPLC—ElCD method include its ease, rapidity of sample preparation, and low cost. A one-step alumina batch extraction has been used successfully for sample preparation of plasma [1, 2], but in applying the technique for urinary catecholamine measurements, a one-step alumina extraction has proven inadequate, because confounding peaks of unknown identity can interfere with the catecholamine peaks [3, 4]. As a result, current HPLC—ElCD methods include additional sample preparation steps [3—5]. The relatively simple, rapid, modified sample preparation described here has dramatically improved the quality of chromatographic recordings for urine catecholamines in this laboratory. The method allows urine-derived eluates to be injected into the same HPLC—ElCD apparatus used for plasma prepared with the alumina extraction, with simultaneous determination of free norepinephrine (NE), epinephrine (E), and dopamine (DA).

EXPERIMENTAL

Sample collection and handling

Urine samples assayed using the HPLC—ElCD technique were obtained from healthy female inpatient volunteers who were not allowed to smoke cigarettes, ingest caffeine-containing or catecholamine-rich foodstuffs, or to take any medications except occasional acetaminophen while on study.

Twenty-four hour total urine collections in 20 ml 6 *N* hydrochloric acid

were refrigerated until the next day. The urine specimens were stored in 20-ml aliquots at −70°C in plastic scintillation vials without other additives.

Reagents

Reagents for the HPLC—ElCD technique included Woelm Super 1 alumina, acid-washed according to the method of Anton and Sayre [6], glacial acetic acid, hydrochloric acid, disodium EDTA, sodium bicarbonate, sodium hydroxide, and sodium acetate (Fisher Scientific, Pittsburgh, PA, U.S.A.); acetonitrile and methanol (Burdick and Jackson Labs., Muskegon, MI, U.S.A.); Tris (Bethesda Research Labs., Bethesda, MD, U.S.A.); heptanesulfonic acid (Fisher or Aldrich); norepinephrine, epinephrine, dopamine, and N-methyldopamine (Sigma, St. Louis, MO, U.S.A.); and distilled, deionized water (Milli-Q, Millipore, Bedford, MA, U.S.A.).

Equipment

The HPLC—ElCD apparatus consisted of a Waters 6000A solvent delivery system, U6K injector, μBondapak C_{18} 30 cm × 3.9 mm reversed-phase stainless-steel column containing 10-μm irregular particulate packing, solvent clarification kit for degassing the mobile phase, and guard column packed with C_{18} Porasil; a Bioanalytical Systems LC4 or LC4A amperometric detector with TL5 glassy carbon electrode; and an LKB or Fisher Recordall strip-chart recorder or Waters Data Module. Waters C_{18} columns and silica Sep-Paks were used for the modified sample preparation as described below.

Mobile phase

The chromatographic mobile phase was prepared as follows. To 1 l water were added 6.8 g sodium acetate, 100 mg EDTA, and 1 g heptanesulfonic acid. The pH was adjusted to 4.8 with 2 *N* hydrochloric acid and the liquid filtered using a vacuum pump and 0.45-μm aqueous filter. Then, 70 ml were discarded and replaced with acetonitrile. The mobile phase was degassed as necessary, usually daily, by stirring under vacuum. The pump was set at 1.0 ml/min, the detector at 10 nA/V, 0.50 V applied potential, and the recorder at 1 V full scale.

Assay steps

A C_{18} Sep-Pak was washed with 10 ml methanol and a silica Sep-Pak with 6 ml of a solution containing 5 ml 1.0 *M* sodium bicarbonate at pH 8.5 and 1 ml 50 g/l EDTA. The C_{18} Sep-Pak was washed with 10 ml water. The C_{18} and silica Sep-Paks were stored in this state overnight in a referigerator prior to urine assay the next day.

The acidified urine was thawed at room temperature. Five ml were passed through the C_{18} Sep-Pak, followed by 1 ml water, and collected in a sample tube. A 2-ml aliquot was mixed with 6 ml of the above described EDTA—bicarbonate solution and the mixture passed through the silica Sep-Pak. The silica Sep-Pak was washed once with 5 ml water. The catecholamines were eluted from the silica by 4 ml of 1% acetic acid. Of this 4 ml, 1 ml was assayed through the batch alumina extraction step. This 1 ml was placed in a 1.5 ml plastic sample tube containing about 10 mg alumina; 400 μl of 1 *M* Tris—20 g/l

EDTA, which had been adjusted to pH 8.6 with hydrochloric acid, were added. The tube was shaken vigorously for 20 min, centrifuged, and the supernatant discarded. The alumina was washed once with 1 ml water and the catecholamines were then desorbed with 100 μl of 0.2 *M* acetic acid. A 50-μl aliquot of the eluate was injected into the HPLC column.

By comparison with a mixture of 100 ng/ml norepinephrine, epinephrine, and dopamine external standards, where 50 μl (5 ng) had been directly injected and where 1 ml (100 ng) was assayed in parallel with the urine, urinary catecholamine excretion was calculated according to the following equation:

Catecholamine excretion (ng per 24 h) = peak height of catecholamine in urine eluate ÷ peak height of external standard × 20 (ng/ml) × volume excreted (ml per 24 h).

Overall recovery through the sample preparation steps was about 40–50% for each of the catecholamines — about 90% through the C_{18} step, 70–75% through the silica step, and 70—75% through the alumina step. No difference in recoveries was obtained among the three catecholamines at any step in the sample preparation.

RESULTS

Fig. 1 shows representative chromatograms of directly injected NE, E, DA, and N-methyldopamine (NMDA) standards and of urine-derived eluates at various stages in the sample preparation. Fig. 1 demonstrates that NE, E, and DA were clearly resolved from each other and that no interfering peaks occurred with the modified sample preparation. Across a total of 181 different

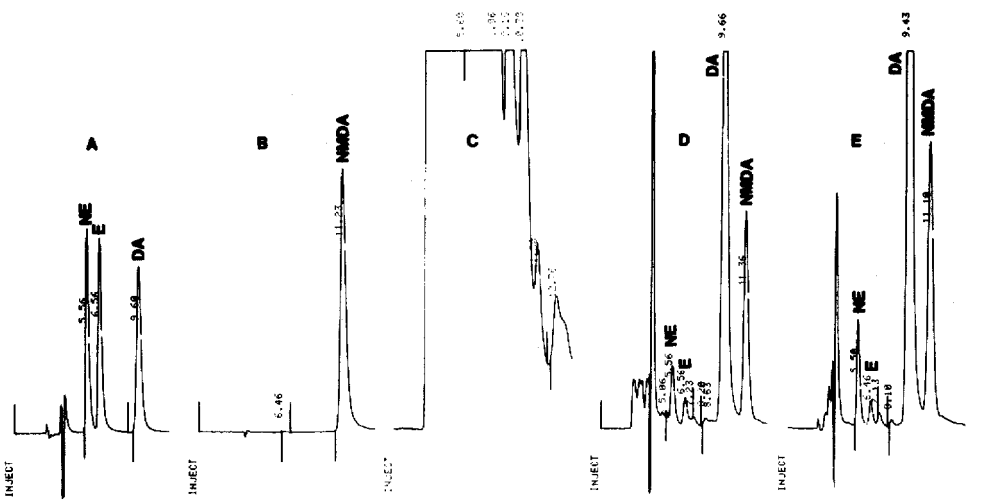

Fig. 1. Chromatographic recordings at various stages of the sample preparation. (A) Injection of 50 μl (5 ng) norepinephrine (NE), epinephrine (E), and dopamine (DA) standards; (B) 1 μl (5 ng) N-methyldopamine (NMDA) standard; (C) 200 μl of partially purified urine sample after C_{18} Sep-Pak; (D) 200 μl of same sample after C_{18} and silica Sep-Paks; (E) 50 μl of same sample after C_{18}, silica, and then alumina batch extraction. Displayed retention times are in hundredths of a minute.

urine specimens, the average norepinephrine excretion per 24 h (± 1 S.D.) was 37 ± 25 μg, epinephrine 7 ± 5 μg, and dopamine 278 ± 187 μg. These results agree well with those obtained by other, older techniques [7]. Adequate chromatography was obtained for all specimens, although occasionally a small peak occurred just after norepinephrine, just after epinephrine, or just before dopamine. After only the C_{18} step, the solvent front invariably was very wide and completely obscured any catecholamine peaks.

DISCUSSION

In this report a modified sample preparation is presented for analysis of urine catecholamines using HPLC—ElCD. Addition of sample purification steps with commercially available C_{18} and silica pre-packed columns prior to alumina batch extraction resulted in excellent chromatographic records, whereas the assay of urine after only an alumina extraction has yielded unreliable results [3, 4]. The modified technique allows injection of urine-derived eluates into the same chromatographic—electrochemical equipment as for plasma-derived eluates, but because of the relatively small recoveries the sample preparation described here for urine can not be used for plasma. Addition of the Sep-Pak columns to the procedure increases the cost of the catecholamine assay by about US$ 2 per sample. Although they can be reused, only new columns were involved in the present study.

Because of the dietary and other restrictions imposed on the healthy women whose urine was assayed, it is possible that some foodstuffs, medications, or disease states may not yield as satisfactory results. The rather large standard deviations as fractions of the mean probably represent real variability in catecholamine excretion rates both within and across individuals, since assays conducted on samples collected for different days in the same individuals resulted in standard deviations of similar magnitude, whereas intra-assay standard deviations among replicates of the same sample were much smaller.

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