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Note

Liquid chromatography assay for 3,4-dihydroxyphenylacetic acid in urine

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In support of our research on tyrosine metabolism, we have been developing a series of specific assay procedures for the various basic, neutral, and acidic metabolites in body fluids and tissue homogenates. The present brief report on urinary 3,4-dihydroxyphenylacetic acid (DOPAC) is intended to complement published work on 3-methoxy-4-hydroxyphenylacetic acid (homovanillic acid, HVA) [1] and the catecholamines [2]. DOPAC and HVA are the major catabolites from the catecholamine dopamine (DA) and as such both must be

DOPAC can be measured by condensation with ethylene diamine and subsequent monitoring of the fluorescent product. This approach has been used by several authors following isolation by various extraction procedures combined with column chromatography or electrophoresis [3—5]. A more recent method employs gas chromatography with electron-capture detection [6]. After isolation of the DOPAC by adsorption chromatography, a volatile derivative is formed by reaction with pentafluoropropionic anhydride.

In this paper we describe a method for the measurement of urinary DOPAC based on liquid chromatography with electrochemical detection. DOPAC is isolated from acidified urine by ethyl acetate extraction and adsorption onto alumina, and is then quantitated by liquid chromatography with electrochemical detection.

MATERIALS AND METHODS

Reagents

The following reagents were used: 6 *M* hydrochloric acid; phosphate buffer, pH 7.4 (1.18 g potassium monobasic phosphate and 8.16 g dibasic phosphate

heptahydrate dissolved in 1 l of distilled water); 0.05 *M* Tris(trihydroxymethylaminomethane) buffer, pH 8.5 (2.21 g Tris hydrochloride and 4.36 g of the free base dissolved in 1 l of distilled water); 0.1 *M* acetate buffer, pH 4.7 (mobile phase); creatinine standard solution (1 mg/ml in distilled water); 1% picric acid in distilled water; 10% (w/v) sodium hydroxide; alumina (acid-washed Woelm W 200 neutral alumina according to the method of Anton and Sayre [7]); and standard urine pool, which is prepared as follows: Combine urine collected from healthy humans. Acidify to pH 2 with 6 *M* HCl and store 20-ml aliquots at −35° in glass scintillation vials. Determine the concentration of DOPAC in the urine pool by the method of standard additions. Analyze the urine pool with and without added DOPAC. Plot the peak height versus amount of DOPAC added and extrapolate to zero peak height to obtain the concentration of DOPAC in the urine pool.

Apparatus

The liquid chromatographic system was a commercial unit based on our earlier work [8,9] using a thin-layer amperometric detector (Bioanalytical Systems, Model LC-2A). DuPont Zipax anion exchange resin was dry packed in a 50 cm × 2.1 mm I.D. glass column (Altex Scientific, Model 251-02). The mobile phase was pumped at a flow-rate of 0.4 ml/min. The detector potential was set at +0.60 V versus an Ag/AgCl reference electrode. Samples were injected using a pneumatically actuated non-metallic 20-μl slider valve (Laboratory Data Control, Model CSV-20).

Procedure

Acidify urine to pH 2 with 6 *M* HCl upon collection and store frozen at −35° prior to analysis. Place 4 ml of urine (pH 2) in a 12-ml glass centrifuge tube, saturate with NaCl (ca. 0.6 g NaCl) and add 4 ml of ethyl acetate. Shake for 10 min on a reciprocal shaker, centrifuge briefly and transfer the ethyl acetate to a 6-in. culture tube using a disposable Pasteur pipet. Repeat the extraction with two additional 4-ml volumes of ethyl acetate. Dry the combined ethyl acetate layers over anhydrous sodium sulfate (ca. 1.5 g). Transfer the ethyl acetate to an acid-washed 12-ml centrifuge tube. Wash the residual sodium sulfate with about 1 ml of ethyl acetate and combine with the original extract.

Concentrate the extract to a volume of about 2 ml under a stream of nitrogen at 25°. Transfer the solvent to a 5-ml conical screw cap vial (Pierce, Reacti-Vial). Wash the centrifuge tube with about 1 ml ethyl acetate and combine with the ethyl acetate concentrate. Evaporate to dryness at room temperature using a stream of nitrogen and add 3 ml of 0.05 *M* pH 8.5 Tris buffer to the residue. Immediately add ca. 70 mg of aluminum oxide and shake on a reciprocal shaker for 15 min. The same quantity of alumina must be added to each vial in order to maintain a reproducible recovery. Allow the alumina to settle, then aspirate the buffer with a fine glass capillary. Wash the alumina twice with pH 7.4 phosphate buffer and once with water, mixing the alumina well each time. Carefully aspirate the final wash to near dryness. Cover the vial with aluminum foil and place a narrow (ca. 5 mm) filter paper wick through a slot in the foil extending into the wet alumina. Place the vials in a vacuum oven and dry in vacuo at 40° for 10 min. If care is taken in the final

aspiration step, then drying the alumina is not necessary. The small amount of water remaining after the final wash does not significantly influence the precision or accuracy of the method.

Elute the DOPAC from the alumina by the addition of 1 ml 1 *M* acetic acid to each vial. Shake the vials on a vortex mixer for 20 sec and allow the alumina to settle. Inject 20 μl of the eluent onto the chromatographic column using the 20-μl slider valve. Measure the peak height of DOPAC and determine the DOPAC concentration by comparison with the peak height obtained for the standard urine pool. Fig. 1 illustrates a representative chromatogram obtained from healthy individuals.

Levels of DOPAC are usually reported relative to the creatinine concentration. Creatinine is determined by the standard picric acid spectrophotometric method [10].

RESULTS AND DISCUSSION

DOPAC was determined in 15 urine samples collected from ten apparently healthy males, ranging from 21 to 30 years of age. DOPAC levels ranged from 0.39 to 1.05 μg per mg creatinine, which is in general agreement with other investigators. The absolute recovery of DOPAC was 48 ± 5.0% relative standard deviation and was found to be constant over a concentration range of at least 1 to 7 μg per ml of urine. The relative standard deviation of the method was found to be ±4.3%. The detector response current was measured from 1 to 100 ng injected and the linear calibration in nA versus ng DOPAC

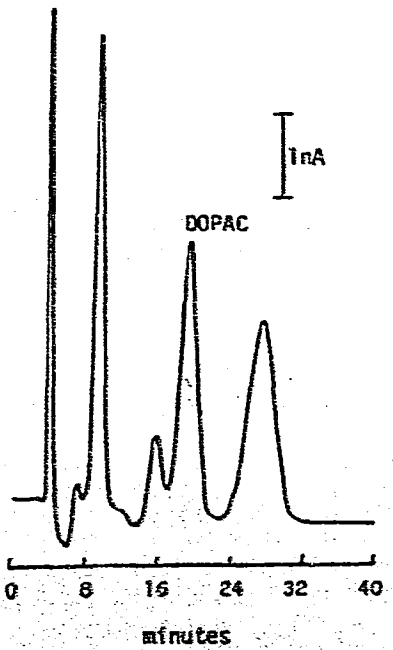

Fig. 1. Chromatogram of DOPAC isolated from a urine pool collected from healthy individuals.

is described by the following equation:

$$\text{Current (nA)} = (1.63 \pm 0.004)\ (\text{ng DOPAC}) - (0.54 \pm 0.06)$$

The detection limit for DOPAC in an aqueous standard was found to be 100 pg.

The present method has advantages of selectivity and sensitivity compared with other solution phase methods. The selectivity of the assay is derived from the combination of four steps, liquid—liquid extraction, liquid—solid extraction, liquid chromatography and electrochemical detection. The initial ethyl acetate extraction removes a majority of the aromatic acids from urine. Only those acids containing a catechol moiety are adsorbed onto the alumina and are then separated by high-performance liquid chromatography. Finally only those compounds oxidizing at +0.6 V versus Ag/AgCl or less are detected. In addition the high-performance liquid chromatographic approach does not require derivatization as do all gas—liquid chromatographic methods.

TABLE I

RETENTION OF CATECHOL ACIDS

Acid	Retention time (min)
3,4-Dihydroxymandelic	13.2
DOPAC	17.0
Protocatechuic	20.8
3,4-Dihydroxy-phenylpropionic	24.1
Gallic	35.0
Caffeic	67.1
Chlorogenic	∞

Several potential interferences have been examined and found to be adequately resolved from DOPAC by the DuPont Zipax (see Table I). Those compounds which elute from the column close to DOPAC are normally found in urine in much lower concentrations and therefore present no problem. If desired, better resolution at the expense of time can be accomplished by a decrease in the mobile phase ionic strength.

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