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Note

Simple and rapid separation of metanephrine and normetanephrine by reversed-phase high-pressure liquid chromatography

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The involvement of catecholamines in cardiovascular, psychiatric and neurological disease states has been the subject of growing interest during recent years [1]. Knowledge in these areas has increased following the development of radioenzymatic techniques sensitive to the picogram quantities found in biological fluids [2]. These methods depend on the enzymatic methylation of catecholamines using tritiated S-adenosyl-L-methionine as methyl donor and either catechol-O-methyl transferase or phenylethanolamine-N-methyl transferase as enzyme. Subsequently, the methylated derivatives are separated by thin-layer chromatography (TLC). The enhanced speed and accuracy of high-pressure liquid chromatography (HPLC) has resulted in attempts to interpose an HPLC, rather than a TLC, step in the assay methodology. One method uses a cation-exchange column [3] but the mobile phase has a pH considerably in excess of that recommended for this type of column and in our experience the column life is very short. Another technique employs ion-pair chromatography but requires critical column preparation, equilibration and temperature control [4].

We describe a simple and rapid method for the separation of normetanephrine and metanephrine using reversed-phase HPLC.

EXPERIMENTAL

Reagents and materials

All reagents necessary to perform the radioenzymatic assay of catecholamines were supplied in the form of a commercial kit (CAT-A-KIT™, Upjohn Diagnostics, Kalamazoo, Mich., U.S.A.). This kit includes a stopping solution containing unlabelled metanephrine, normetanephrine and methoxytyramine in the concentration of 4 m*M* to act as carrier for the radiolabelled reaction products. The water used for chromatography was glass distilled. Methanol used in the chromatography was "distilled in glass" quality, purchased from Burdick and Jackson (Muskegon, Mich., U.S.A.). Heptane sodium sulphate was purchased from Eastman Kodak (Rochester, N.Y., U.S.A.). Omnifluor was purchased from New England Nuclear (Los Angeles, Calif., U.S.A.). All other solvents and reagents were reagent-grade quality. The amyl alcohol was prewashed with 5% phosphoric acid and rinsed with water.

Sample preparation

Only 20 μl of plasma are required for this assay. Norepinephrine and epinephrine are radioenzymatically converted to normetanephrine and metanephrine using tritiated S-adenosyl-L-methionine as the methyl donor and catechol-O-methyl transferase as the enzyme. The reaction is allowed to proceed for 1 h at 37° in the presence of buffer containing 2-amino-2(hydroxymethyl)-1,3-propanediol (tromethamine), ethylene glycol-bis(β-aminoethyl ether)-N,N′-tetraacetic acid (EGTA) and magnesium chloride. The reaction is terminated with stopping solution containing borate buffer, unlabelled metanephrine, normetanephrine and methoxytyramine. Extraction is performed into 2 ml of a mixture of toluene in amyl alcohol (3:2) with back extraction into 20 μl 0.1 *N* sulphuric acid. The entire acid phase was injected into the chromatograph.

Chromatography

A Varian 8500 dual-pump high-pressure liquid chromatograph was used, fitted with a Varian Micro-Pak MCH-10 (Monomeric C18 bonded phase) reversed-phase column (25 cm × 2.0 mm I.D.). Pump A contained a 0.01 *M* solution of 1-heptanesulphonic acid sodium salt in water adjusted to pH 3.5 with glacial acetic acid. Pump B contained the same concentrations of salt and acid in methanol. Both solvents were filtered through Whatman No. 2 paper using a vacuum. An isocratic mixture containing 10% B with a flow-rate of 50 ml/h and a precolumn pressure of 1500 p.s.i. was used for analysis. A Vari-Chrom® detector was used to measure absorbance at 280 nm using an 8-nm band width. Chromatograms were recorded on a Varian 9196 recorder with a 100 mV span set at a chart speed of 1.0 cm/min. Effluent was collected under each peak from the outlet of the detector.

Subsequent analysis

The samples were brought to pH 10 with 0.05 *M* ammonium hydroxide and then oxidised with sodium meta periodate [2]. Following acidification with 0.1 *M* acetic acid the oxidation products were extracted into 8 ml of toluene. The aqueous phase was frozen in an acetone—dry-ice bath and the toluene

decanted into a scintillation vial containing 0.6 ml Omnifluor^R in the concentration 10 g of Omnifluor per 100 ml toluene. The scintillating fluid was brought to a volume of 15 ml with additional toluene before counting.

RESULTS AND DISCUSSION

Under the chromatographic conditions described, the normetanephrine, metanephrine and methoxytyramine peaks occur at 3.8, 6.0 and 9.5 min. As shown in Fig. 1, the chromatographic separation is excellent and allows accurate collection of effluent.

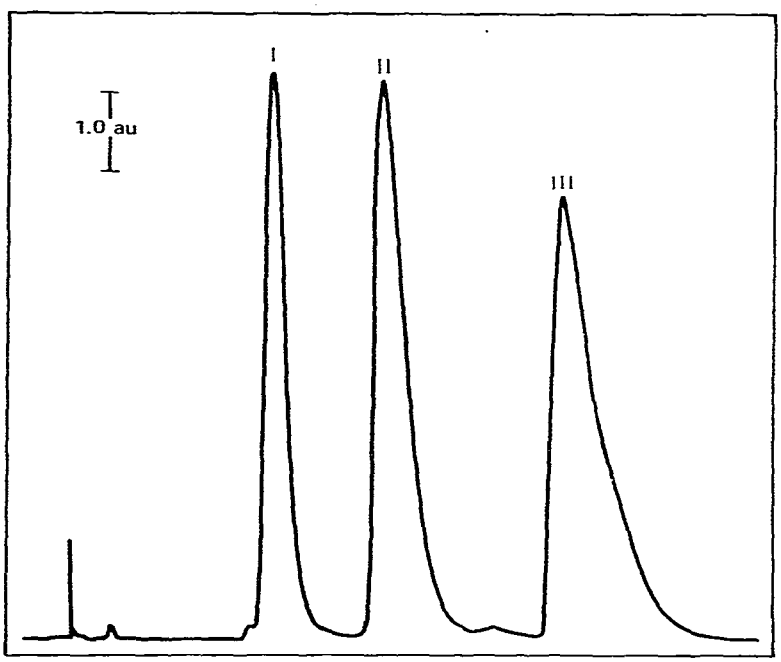

Fig. 1. Chromatogram of methylated catecholamines under the conditions described in the text. I, Normetanephrine 3.8 min; II, metanephrine 6 min; III, methoxytyramine 9.5 min.

Although the methoxytyramine peak is well defined, an unidentified radioactive product of the enzymatic reaction contaminates the effluent collected under this peak and prevents the accurate measurement of very low concentrations of dopamine. However, this method has given reliable results for both epinephrine and norepinephrine when used to analyze plasma containing known concentrations (Table I).

The disadvantage of using TLC in this as in other assays rests on the relatively poor resolution obtained because of tailing between the zones. This leads to coefficients of variation as high as 15% for interassay data [2]. HPLC has the advantage of high resolution which in our experience has resulted in an interassay coefficient of variation of 4%.

The enhanced accuracy of HPLC has led to several attempts at interposing an HPLC step in the radioenzymatic assay of catecholamines. The method we describe has the advantage of simplicity and reliability.

TABLE I

DATA OBTAINED USING METHOD DESCRIBED IN THE TEXT

	Normetanephrine (pg/ml)	Metanephrine (pg/ml)
Actual	840	580
Calculated	810	579
Actual	680	485
Calculated	662	463

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