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ESTIMATION OF CATECHOLAMINES BY ION-EXCHANGE CHROMATOGRAPHY ON ASAHIPAK ES-502C, USING GLYCYLGLYCINE AS THE POST-DERIVATIZING AGENT

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SUMMARY

The estimation of catecholamines in human urine was carried out by ion-exchange chromatography on a column of a weakly acidic ion exchanger with an hydrophilic matrix. The catecholamines were first adsorbed onto Amberlite CG-50 (buffered at pH 6.5 with 0.4 *M* phosphate buffer) and selectively eluted by 0.66 *M* boric acid solution. They were then separated from impurities that responded to fluorometric detection by isocratic elution from a column of Asahipak ES-502C, a cross-linked vinyl alcohol copolymer with carboxymethyl groups, at 60°C. The mobile phase was 0.05 *M* sodium succinate buffer pH 5.25 containing 0.015 *M* borate and 0.5 m*M* ethylenediaminetetraacetate. Isoproterenol was used as the internal standard; epinephrine, norepinephrine, isoproterenol and dopamine were eluted in this order. One sample could be analyzed every 35 min. The detection limits were 0.2 ng for epinephrine and norepinephrine, 0.6 ng for dopamine. The elution pattern was quite reproducible; the elution volumes of the catecholamines had not changed after 500 determinations.

INTRODUCTION

The separation of catecholamines has been performed by ion-exchange[1–6] or ion-pair chromatography[7–10]. Reversed-phase chromatography was particularly suitable for the separation of fluorescent derivatives of catecholamines[11–14]. Eluents containing crown ethers have also been used[15]. However, when these chromatographic methods were used for the separation of urinary catecholamines, impurities to which the electrochemical detector responded or whose fluorescence was excited by the light used for fluorometric estimatioin sometimes overlapped with the peaks of catecholamines.

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Since hydrophobic interaction of the impurities with the stationary phase may be the main cause of this interference, we used a cation exchanger with an hydrophilic matrix for the separation of catecholamines in human urine. Complete separation of catecholamines from impurities that respond to the fluorometric estimation was achieved.

EXPERIMENTAL

Reagents

Epinephrine bitartrate, norepinephrine bitartrate, dopamine hydrochloride, isoproterenol hydrochloride and glycylglycine were purchased from Sigma (St. Louis, MO, U.S.A.). Other chemicals were of analytical grade and obtained from Yashima Pharmaceutical Co. (Osaka, Japan). Stock solutions of catecholamines (1 mg/ml) were prepared in 0.01 *M* hydrochloric acid, and they were diluted in a 0.027 *M* succinate–0.42 *M* borate buffer (pH 5.25), containing 3 m*M* ethylenediaminetetraacetate and β-thiodiglycol (0.1%, w/v) to give a standard solution containing 50 ng of epinephrine, 50 ng of norepinephrine, 125 ng of dopamine and 100 ng of isoproterenol per ml.

Equipment

A constant-flow pump (Jasco, Model Trirotar III) was used to pump buffer through the chromatographic column. A dual-head pump (Jasco, Model SP-024-2) was used to pump reagents and mix them with the eluate. A fluorometer (Jasco, Model FP-115), equipped with a flow cell (volume, 7 μl), was used to measure fluorescence. Samples were injected by an automatic injector (Kyowa Seimitsu, Model KSST-60J).

Preparation of Amberlite CG-50 column

Amberlite CG-50 (Type 2) was graded according to size and washed, as described previously[5]. The sodium ion form of the resin (particle size 100–140 μm) was transferred with three volumes of water into a beaker, and the suspension was adjusted to pH 6.7 with a solution of 0.4 *M* sodium dihydrogen phosphate. Then the suspension was poured into a sintered glass filter and washed with a 0.4 *M* phosphate buffer pH 6.5 until the pH of the washings was 6.5. The buffered resin was poured into a tube (10 × 0.5 cm I.D. with a 5-ml reservoir) with 0.4 *M* phosphate buffer pH 6.5 and allowed to settle under gravity to form a resin bed, 1.0 ml in volume. The column was washed with 1 ml of deionized water before use.

Separation of catecholamine fraction from human urine

A 1.0-ml portion of filtered urine was mixed with 0.2 ml of a 5% solution of disodium ethylenediaminetetraacetate, 0.2 ml of 1% solution of ascorbic acid and 200 ng of isoproterenol in 1.0 ml of 0.01 *M* hydrochloric acid, and the mixture was adjusted to pH 6.2–6.3 with 1 *M* sodium hydrogen carbonate solution. The mixture was then applied to an Amberlite CG-50 column, which was washed with 3 ml of deionized water and then with 0.8 ml of 2/3 *M* boric acid solution. A further 1.25 ml of boric acid solution were used to elute catecholamines from the column, the eluate being collected in a test-tube containing 0.70 ml of 0.08 *M* succinic acid and 0.05 ml each of 5% disodium ethylenediaminetetraacetate solution and 5% β-thiodiglycol solution. The catecholamine fraction was stored in a refrigerator.

Chromatographic separation and fluorometric determination

A 0.2-ml of aliquot of the sample solution was injected into a 10 × 0.76 cm I.D. column packed with Asahipak ES-502C, (a cross-linked vinyl alcohol copolymer with carboxymethyl groups, column temperature kept at 60°C). The mobile phase (pH 5.25, 0.05 *M* succinate–0.015 *M* borate–0.5 m*M* ethylenediaminetetraacetate) was pumped at a rate of 1.0 ml/min and the eluate was mixed with reagents A and B. Reagent A was a solution of 0.1 *M* glycylglycine pH 6.5 containing 0.05 *M* boric acid, 3 m*M* zinc sulphate and 0.2 *M* tartaric acid; reagent B was a 0.25 *M* potassium borate buffer pH 9.2 containing hexacyanoferrate(III) (0.01%, w/v). Each reagent was pumped at a flow-rate of 0.35 ml/min with a dual-head pump and mixed by using a T-shaped connector. The mixture of reagents and the eluate from the column was heated at 90°C in a PTFE tube (30 m × 0.5 mm I.D., 1.5 mm O.D.), immersed in a water-bath. The fluorescence was measured with a fluorometer (Jasco, Model FP-115).

RESULTS AND DISCUSSION

The elution patterns of standards and of a urine sample are shown in Fig. 1. The pH of the mobile phase was 5.25 and was optimal for the separation of four catecholamines eluted in the order: epinephrine (E), norepinephrine (N), isoproterenol (I) and dopamine (D). The use of an eluent with higher pH shifted the position of the peak of I between those of E and N, and a higher concentration of borate in the eluent reduced the elution volumes of the peaks of these catecholamines and also reduced the separation of the peaks of E, N and I. The urine sample contains a higher concentration of borate (0.42 *M*) than that in the mobile phase (0.015 *M*), but the higher concentration did not have an adverse effect on the elution pattern of catecholamines because the sodium ion concentration of the sample (0.04 *M*) is lower than that of the mobile phase (0.075 *M*). Although the resolution of the peaks of these catecholamines was better at 50°C, separation at 60°C was necessary for analysis of some samples.

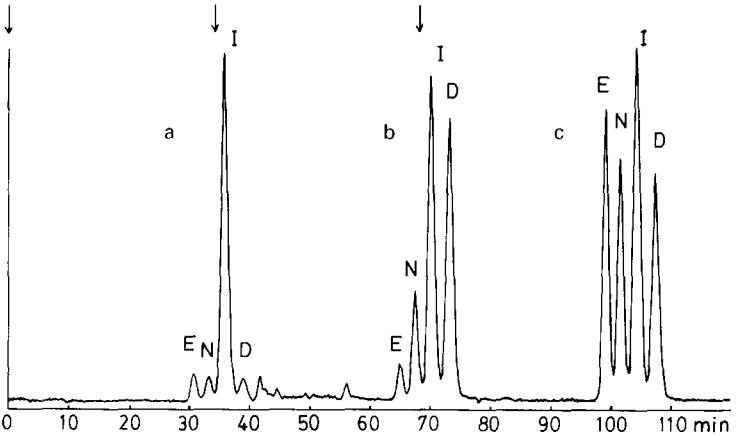

Fig. 1. Elution pattern of catecholamines. The arrows indicate the time of injection. a, 1 ng of E and N, 20 ng of I and 2.5 ng of D; b, catecholamine fraction of human urine; c, 10 ng of E and N, 20 ng of I and 25 ng of D.

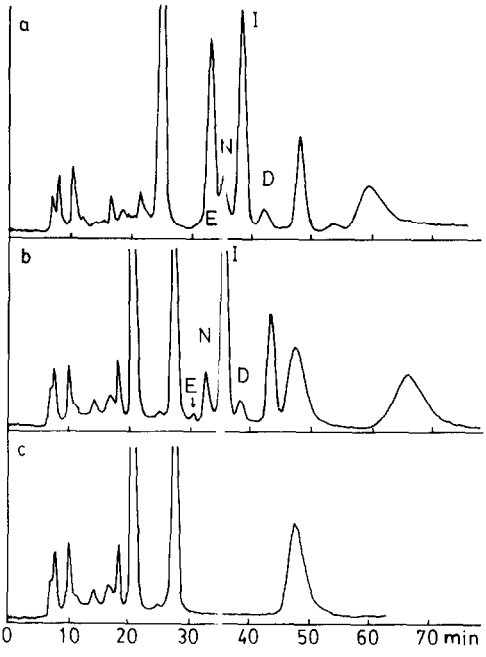

Fig. 2. Elution patterns of catecholamines in a urine sample. a, Separation at 50°C. Peak of E overlapped with the large unidentified peak just ahead of the peak of N. b, Separation at 60°C. E was eluted as the small peak between the large unidentified peak and the peak of N. c, Separation at 60°C, with water instead of reagent A. Note that the peaks of the catecholamines have disappeared.

As is shown in Fig. 2a, at 50°C the peaks of E and N overlapped with those of impurities, but the impurities were eluted before E at 60°C (Fig. 2b). When water was used instead of reagent A, the peaks due to the catecholamines disappeared completely (Fig. 2c). Therefore, the selectivity of the glycylglycine method was confirmed. The presence of zinc sulphate in reagent A, as described previously[5], increased the fluorescence intensity of the products derived from dopamine, and a further increase in intensity was observed upon addition of 0.2 *M* tartrate to reagent A.

A linear relationship between the peak height and the amount of the amines added to the column was obtained over the range 0.5–100 ng for epinephrine and norepinephrine and 1.5–150 ng for dopamine. Samples could be analyzed every 35 min, and even after 500 determinations the elution pattern was quite reproducible. Based on seven determinations of 1.0-ml aliquots of the same urine sample, the mean amounts of E, N and D per millilitre of urine were calculated to be 13.6 ± 0.34 (S.D.), 46.2 ± 1.12 and 316 ± 9.23 ng respectively. When 100 ng each of E and N and 250 ng of D were added to a urine sample and the procedure described above was carried out, the recoveries were 97.7 ± 2.66 (S.D.), 96.5 ± 3.43 and 97.0 ± 2.03% respectively (six determinations).

The results indicate that Asahipak ES-502C, a cross-linked vinyl alcohol copolymer, is suitable for the estimation of catecholamines in human urine.

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