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POSTCOLUMN DERIVATIZATION OF CATECHOLAMINES WITH 2-CYANOACETAMIDE FOR FLUORIMETRIC MONITORING IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Catecholamines in the eluates of high-performance liquid chromatography were reacted with 2-cyanoacetamide in borate buffer in flow analysis mode, and the intensity of fluorescence developed was recorded. Under the optimum conditions (column: Hitachi 3011 C, 25 cm × 2.6 mm I.D., 45°C; eluent: 0.05 *M* KH_2PO_4 containing 0.05% H_3PO_4, 0.60 ml/min; reagent solution: a mixture of 1% 2-cyanoacetamide, 0.50 ml/min, and a 0.60 *M* H_3BO_3—KOH buffer, 1.0 ml/min; size of reaction coil: 5 m × 0.5 mm I.D.; reaction temperature: 100°C; wavelengths for detection: 383 nm for excitation and 486 nm for emission), this method allowed simultaneous determination of 5—500 pmol catecholamines with high reproducibility. The lower limits of detection (signal-to-noise ratio = 2) for epinephrine, norepinephrine and dopamine were 0.28, 0.11 and 0.098 pmol, respectively. Some applications of the analysis of urinary and serum catecholamines are also presented.

INTRODUCTION

Biochemical and clinical investigations of catecholamines have made great progress owing to the development of sensitive high-performance liquid chromatography (HPLC). Although electrochemical detection is currently used for monitoring catecholamines [1], fluorimetric detection based on postcolumn derivatization still plays an important role, because of its stability and the durability of the monitoring system. The most widely used method for fluorimetric detection is the method based on postcolumn oxidation to trihydroxyindole (THI) derivatives and their fluorimetric monitoring [2]. However, this method has the disadvantage that its sensitivity for dopamine (DA) is approximately two orders of magnitude lower than for epinephrine (E) and norepinephrine (NE). The ethylenediamine (ED) method [3] shows rather uniform sensitivity to all catecholamines, but it is less sensitive than the THI method.

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Recently, we found that the condensation reaction with 2-cyanoacetamide is also applicable to fluorimetric determination of catecholamines. The results obtained for manual determination [4] demonstrated its high, uniform sensitivity, as well as simplicity of procedure. Therefore, we have applied this reaction to fluorimetric monitoring of catecholamines in HPLC.

EXPERIMENTAL

Materials

A sample of 2-cyanoacetamide was obtained from Kanto Kagaku (Tokyo, Japan) and used without further purification. Reagent grade samples of E (bitartrate), NE (bitartrate) and DA (hydrochloride) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan), Wako (Osaka, Japan) and Nakarai (Kyoto, Japan), respectively. All other chemicals were of the highest grade commercially available.

The samples of urine and serum were collected from a 24-year-old healthy man and were immediately subjected to clean-up treatment with aluminium oxide.

Apparatus

Fluorescence spectra were recorded on a Shimadzu RF-500 spectrofluorimeter using a 1 × 1 cm quartz flow cell.

Separation of catecholamines by HPLC

A Hitachi 635 A high-performance liquid chromatograph, equipped with a jacketed stainless-steel column (25 cm × 2.6 mm I.D.) packed with Hitachi 3011 C resin (particle size, 10—25 μm), was used for the separation of catecholamines. The column was maintained at 45°C by circulating warm water and eluted with 0.05 *M* dipotassium hydrogen phosphate containing 0.05% phosphoric acid at a flow-rate of 0.60 ml/min. This separation system is a modification of the system used for Hitachi catecholamine analyzers. The solutions of catecholamine samples were introduced onto the column via a Rheodyne 20-μl loop injector.

Postcolumn derivatization

Atto SF-2396 twin-piston pumps were used to supply the reagent solution. One pump head was used for pumping an aqueous 1% solution of 2-cyanoacetamide and the other for pumping a borate buffer. These solutions were mixed by a three-way connector, and the resultant reagent solution was introduced into the stream of the eluate via another three-way connector. The flow-rates of the aqueous reagent solution and the buffer were 0.50 and 1.0 ml/min, respectively.

The postcolumn derivatization was performed in a PTFE coil with an inner diameter of 0.5 mm, which was immersed in a glycerol bath thermostated at 100±1°C. The fluorescence intensity was measured with a Hitachi 650 LC fluorimetric detector equipped with a 90-μl quartz flow cell. All tubing in the derivatization system was PTFE with an inner diameter of 0.5 mm.

Clean-up of urine samples

To a 5-ml portion of urine was added 2 *M* hydrochloric acid (5.3 ml), and the mixture was heated for 20 min at 100°C. After the mixture was cooled to room temperature, 0.05 *M* disodium ethylenediaminetetraacetate (1 ml) was added, followed by ammonia water to adjust the pH to 8.5. Aluminium oxide (Wako, 200 mesh, 500 mg) was added to the resultant solution, and the mixture was shaken for 10 min. Then the mixture was filtered by a pencil column (5 cm × 8 mm I.D.) carrying a Millipore filter. The aluminium oxide in the column was subsequently washed with water (10 ml), followed by 0.3 *M* acetic acid (5 ml). The acetic acid eluate was analyzed for catecholamines.

Clean-up of serum samples

The procedure of Itano [5] was slightly modified. Activated aluminium oxide (100 mg) was suspended in 1 *M* Tris—HCl buffer (pH 8.7, 1 ml), and to this suspension was added a serum sample (1 ml). The mixture was stirred and allowed to stand for 10 min. The supernatant was discarded, and the precipitates were washed three times with 5-ml portions of water, followed by methanol (3 ml). The precipitates finally obtained were dried under reduced pressure, and catecholamines adsorbed on the precipitates were percolated with 4 *M* acetic acid (3 ml). The percolate (2.7 ml) was evaporated to dryness under reduced pressure, and the residue was dissolved in water (90 μl). A 10-μl portion of the solution was subjected to catecholamine analysis.

RESULTS AND DISCUSSION

Characteristics of the fluorescence reaction

The generation of fluorescence from catecholamines and 2-cyanoacetamide is considered to be due to the production of nitrogenous bicyclic compounds by condensation of the diphenol group with the reagent, followed by dehydrative cyclization [6]. The presence and absence of the hydroxyl and N-methyl groups in the aliphatic chain are responsible for slight variation of the optimum pH and wavelengths of excitation and emission maxima among catecholamines. In the established procedure for manual determination [4] an aqueous solution of a catecholamine sample, an aqueous 1% solution of 2-cyanoacetamide and a 0.3 *M* borate buffer were mixed and the mixture was heated to generate fluorescence. The optimum pH of the buffer to be added was 12.0, 10.0 or 11.0 for E, NE or DA, respectively. The wavelengths for detection were (excitation/emission) 380/483, 361/460, or 362/435 nm, respectively.

pH dependence of postcolumn derivatization

Fig. 1 shows the relationship between pH of waste fluid and peak response for each catecholamine species. Each catecholamine was detected at three different wavelengths mentioned above for manual determination.

The maximum point for E was observed at about pH 11, when monitored at 380/483 nm. For other wavelengths the peak response continued to increase with increasing pH values, but it did not exceed the maximal value for 380/483 nm over the whole pH range. Although there was a slight difference of optimum pH between HPLC (approx. 11) and manual determination (12.0),

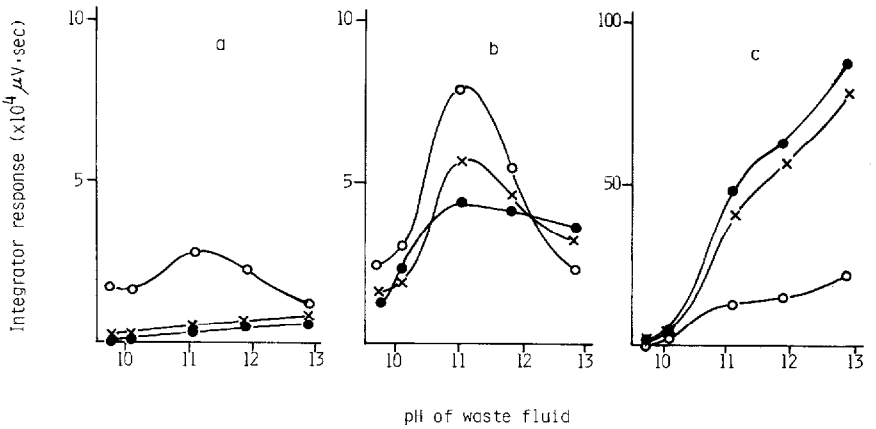

Fig. 1. Relationship between pH of waste fluid and peak response for (a) epinephrine, (b) norepinephrine and (c) dopamine. (○) Obtained at 380 (excitation)/483 (emission) nm; (×) obtained at 361/460 nm; (●) obtained at 362/435 nm. Sample scale: 50 pmol of each.

it should be noted that the pH for HPLC was that of the waste fluid and the value for manual determination was that of the buffer to be added. Because the HPLC eluate was acidic this would explain the difference of pH.

For monitoring of NE, unexpected phenomena were observed. Firstly, the peak response obtained at 361/460 nm, where the maximum intensity was obtained for NE in the manual method, was smaller than that obtained at 380/483 nm. Secondly, the optimum pH (approx. 11) was higher than that (10.0) for manual determination. The value for HPLC should be somewhat lower considering the acidity of the eluate. In order to clarify these discrepancies, NE was reacted with 2-cyanoacetamide both in a borate buffer and a mixed borate—phosphate buffer having an identical pH value. These systems correspond to manual determination and HPLC, respectively. The result was that the former system gave only one fluorescence peak at 460 nm with the excitation maximum at 361 nm, as expected, but in the latter system two fluorescence peaks occurred at 451 and 466 nm, with excitation maxima at 362 and 371 nm, respectively. This diversity of fluorescence formation may account for the unexpected pH—peak response profile of NE.

The peak response of DA increased rapidly up to a pH of approx. 11. Thereafter, the rate of increase was rather reduced. The curve obtained at 362/435 nm, the wavelengths of DA for manual determination, was the uppermost over this pH range. These observations conform to the results obtained for manual determination.

Length of reaction coil

Reaction coils of various lengths were examined for derivatization, and the best result was obtained for the length of 5 m, when a PTFE coil with an inner diameter of 0.5 mm was used. It took 35 sec for a sample to pass through this coil when the flow-rates of the eluate and the reagent solution were controlled at 0.60 and 1.50 ml/min, respectively. With longer coils separation of peaks was incomplete due to spreading. With shorter coils peaks were smaller.

Recommended conditions for monitoring catecholamines

Although pH and wavelength for detection can be selected for each catecholamine species in manual determination, they should be fixed to common values in HPLC. We chose a value of 11 for the pH of the waste fluid. This value could be obtained by using a borate buffer containing 0.60 *M* boric acid and 0.75 *M* potassium hydroxide as reagent buffer. The selected wavelengths were 383 nm (excitation) and 486 nm (emission). Fig. 2 shows the chromatogram obtained for an equimolar mixture of authentic catecholamines under these conditions.

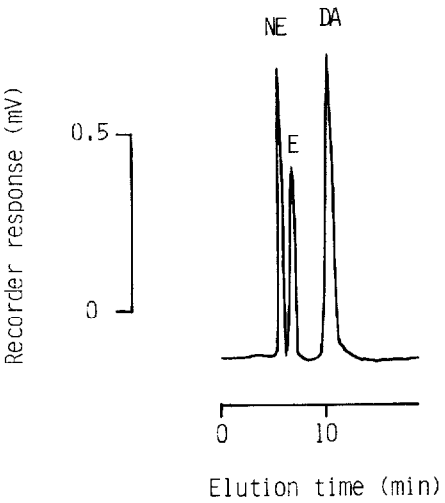

Fig. 2. Chromatogram of an equimolar mixture of epinephrine (E), norepinephrine (NE) and dopamine (DA), obtained by fluorimetric monitoring with 2-cyanoacetamide. Column, Hitachi 3011 C (25 cm × 2.6 mm I.D.); column temperature, 45°C; flow-rate of eluate, 0.60 ml/min; reagent solution for postcolumn derivatization, a mixture of an aqueous 1% solution (0.50 ml/min) of 2-cyanoacetamide and 0.60 *M* H_3BO_3—0.75 *M* KOH buffer (1.0 ml/min); wavelengths for detection, 383 nm (excitation)/486 nm (emission); sample scale, 20 pmol.

Calibration curves

As can be seen in Fig. 3, the calibration curves for all catecholamines showed good linearity for sample amount in the range 5 to at least 500 pmol.

Sensitivity

The lower limits of detection of E, NE and DA were 0.28, 0.11 and 0.098 pmol, respectively, for a signal-to-noise ratio of 2. The sensitivity for DA was approximately 100 times higher than with the THI method, and the sensitivity for E and NE was approximately the same as with the THI method.

Reproducibility

The coefficient of variation obtained for ten determinations of E, NE and DA at the 50 pmol level was 3.1%, 3.9% and 3.2%, respectively, indicating that the present method is satisfactorily reproducible. The values at the 5 pmol level were rather high (6.8%, 6.6% and 4.9%, respectively), but they were within the limits for practical analysis of biological samples.

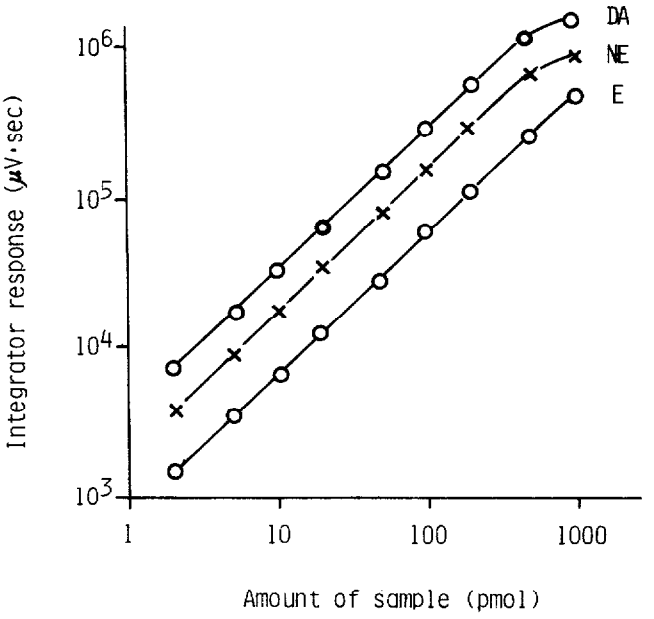

Fig. 3. Calibration curves of epinephrine (E), norepinephrine (NE) and dopamine (DA).

Interference

In the fluorescence reaction of catecholamines with 2-cyanoacetamide there was interference from the presence of reducing carbohydrates. The relative molar intensities of aldoses to DA were in the range 0.01—0.04 when measured by the manual method using a borate buffer of pH 11.0. However, the interference in HPLC was not very serious when the molar ratio of total reducing carbohydrates to catecholamines was less than 100, because the peak of reducing carbohydrates eluted fast and was well separated from those of catecholamines. Interference by other biological substances such as amino acids, carboxylates, proteins, nucleic acids and related compounds, as well as inorganic salts in body fluids, was negligible at equimolar levels.

The detailed data obtained for manual determination have been reported [4].

Analysis of urinary catecholamines

Direct injection of a urine sample gave a sharp peak of DA, but the peaks of E and NE were superimposed on a fast-eluting tailing peak arising from reducing carbohydrates, since the molar ratio of total urinary reducing carbohydrates to urinary catecholamines was in the range 100—1000 [6]. Therefore, urine samples were pretreated by a simple procedure with commercial aluminium oxide. Fig. 4 shows an example of chromatograms obtained for a pretreated urine sample. The volume of the sample injected onto the column was equivalent to 10 μl of untreated urine. The major urinary catecholamine was shown to be DA (276 ng/ml). The concentrations of E and NE were 14 and 24 ng/ml, respectively. All these values were consistent with the reported values of normal urinary catecholamine levels [7].

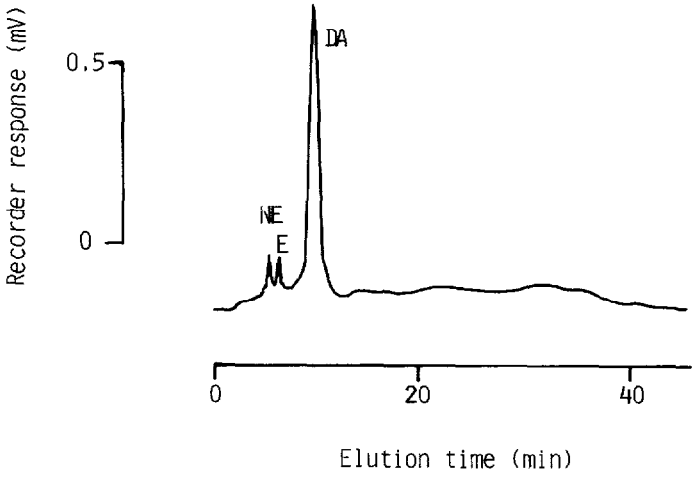

Fig. 4. Analysis of urinary catecholamines of a normal man by fluorimetric monitoring with 2-cyanoacetamide. Injected sample volume was equivalent to 10 μl of urine. E = epinephrine; NE = norepinephrine, DA = dopamine.

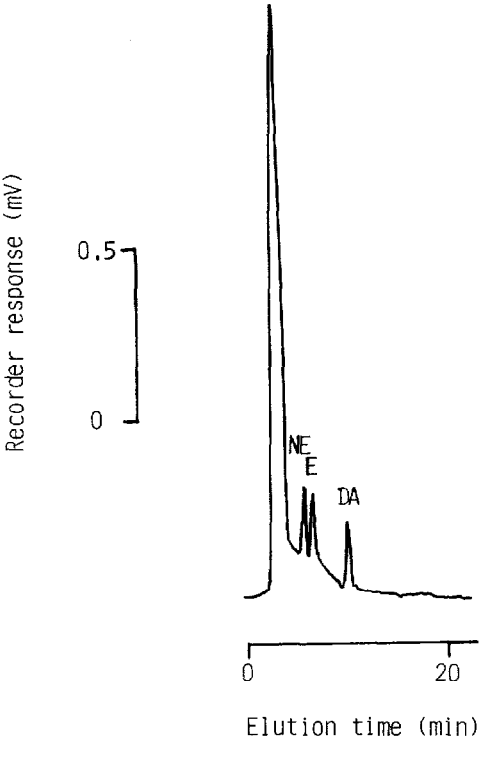

Fig. 5. Analysis of serum catecholamines of a normal man by fluorimetric monitoring with 2-cyanoacetamide. Injected sample volume was equivalent to 100 μl of serum. E = epinephrine, NE = norepinephrine, DA = dopamine.

Analysis of serum catecholamines

Because the serum D-glucose level (approx. 5 μmol/ml) was much higher than the catecholamine level (1—10 pmol/ml), the interference by this aldose was serious. Accordingly, serum samples were pretreated by a modification of the method of Itano [5] by using activated aluminium oxide. Fig. 5 shows an example of chromatograms obtained for pretreated serum samples.

The injected volume was equivalent to 100 μl of serum. Peaks of E, NE and DA were easily recognized and quantitated, the concentrations being 0.9, 2.6 and 2.6 ng/ml, respectively. All these values were slightly higher than those reported in the literature [7] (E = 0.77±0.09 ng/ml; NE = 1.69±0.20 ng/ml; DA = 1.16±0.06 ng/ml), probably because the blood sample was collected from a patient in a rather excited state.

The foregoing results indicate that the postcolumn derivatization of catecholamines to fluorescent products with 2-cyanoacetamide may offer a simple method for their monitoring by HPLC. The high and uniform sensitivity of the method for all naturally occurring catecholamines should be evaluated for the simultaneous analysis of biological samples, especially body fluids.

REFERENCES

1 P.T. Kissinger, C.S. Bruntlett, G.C. Davis, L.J. Felice, R.M. Riggins and R.E. Shoup, Clin. Chem., (1977) 1449.
2 Y. Yui, T. Fujita, T. Yamamoto, Y. Itokawa and C. Kawai, Clin. Chim. Acta, 26 (1980) 194.
3 T. Seki, J. Chromatogr., 155 (1978) 415.
4 S. Honda, Y. Araki, M. Takahashi and K. Kakehi, Anal. Chim. Acta, in press.
5 Y. Itano, unpublished results.
6 S. Honda, unpublished results.
7 A. Mori and H. Kishikawa, Jap. J. Clin. Chem., 4 (1975) 34.