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The Microanalysis of Rat Brain Noradrenaline and Dopamine by High Performance Liquid Chromatography*)

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ABSTRACT

A simple, sensitive and reproducible microanalysis of noradrenaline and dopamine in the rat brain by high performance liquid chromatography with fluorometric detection by the o-phthalaldehyde (OPA) method is described. At the picomoler level, the recovery of catecholamines from the alumina column as the clean up procedure was 77.8±4.0% for noradrenaline, 61.0±2.9% for dopamine and 72.8±2.9% for 3,4-dihydroxybenzylamine (DHBA) as an internal standard (mean±SD, n=10). The variation coefficients of reproducibility of the present analytical method (total of clean up, separation and detection method) were 4.3% for noradrenaline and 3.0% for dopamine of the rat brain. The detectable limits of this method were 10 picomoles for noradrenaline and 20 picomoles for dopamine which corresponded to 1.7 ng and 3 ng, respectively. Catecholamine contents of the whole rat brain with present method were 2.027±0.234 nmol/g wet tissue for noradrenaline and 4.333±0.365 nmol/g wet tissue for dopamine (mean±SD, n=5).

INTRODUCTION

Many detection methods have been reported for analysis of cathecholamines, in which trihydroxyindole (THI)[1,4)] or ethylendiamine (ED)[13)] method with fluorometric detection have been frequently used. Recently many advanced high sensitive methods by high performance liquid chromatography (HPLC) have been performed to detect brain catecholamines using the electrochemical detector[5,7,10)] or fluorescamine reagent with the fluorometric detector[6)]. In order to obtain more accurate and reproducible data, however, some adequate clean up procedures and also simple, sensitive and reproducible detection method are still required for the microanalysis of biogenic amines.

This report presents two points. One is to simplify clean up techniques using a small alumina column with 3, 4-dihydroxybenzylamine (DHBA)[5-7,10)] as an internal standard. The other is to present the rapid, sensitive and repoducible microanalsis of the rat brain nor--adrenaline and dopamine by HPLC using o-phthalaldehyde (OPA) method[2,3)].

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MATERIALS AND METHODS

Materials; Dopamine hydrochloride, noradrenaline, 3, 4-dihydroxybenzylamine and o-phthalaldehyde were obtained from Sigma chemical Co. (USA). Alumina was obtained from E. Merk (Germany) and purified according to the directions by Anton and Sayre[1] (1962). All other chemicals were reagent grade and used without purification.

Preparation of samples; Adult male Wistar rats weighing 200 to 250 g were used after twenty four-hour starvation. The whole rat brain was rapidly removed after decapitation and then immediately weighed and homogenized with about 10 fold ice cold 5% trichloroacetic acid (TCA) containing $25/8 \times 10^{-7}$ M DHBA as the internal standard for catecholamine and 1% mercaptoethanol (MeOH) as the anti-oxidant. After centrifugation at $1,500 \times g$ for 15 min at 0°C, the supernatant was kept at −10°C until analysis.

Alumina adsorption of catecholamines; Making a simple alumina column, 1.2 g of purified alumina was transfered into a 2.5 ml polyethylen syringe (TERUMO Co. Ltd., Tokyo). Each 800 μl supernatant was added 150 μl of 1 N NaOH and 2 ml of 1 N sodium bicarbonate to adjust pH near 8.5. This procedure is very easy and reproducible to adjust the pH value of the sample solution. Then the mixture solution was quickly and carefully applied on the alumina column. The alumina column was washed out with 6 ml of 0.2 M phosphate solution (pH 8.5) and then 2 ml of distilled water. Then adsorbed catecholamines on the alumina column were eluted with 1 ml of 1 N acetic acid into a polyethylen tube. The eluate was mixed with 150 μl of 1% EDTA and 2% sodium hydrosulfate to minimize oxidative destruction of catecholamines. After the eluate was lyophilized, the dried powder was dissolved in 50 μl of the buffer (0.2 N citrate buffer pH 3.25, 0.5 % thiodiglycol as anti-oxidant).

Each 20 μl of samples was injected into the analytical ion-exchange column of HPLC through Rheodyne sample injector (model 7125, California), and then some remaining eluates were kept at −10°C for the study of the stability. Each sample of supernatant of the brain homogenate was analyzed three times and the result was shown as the mean of the three values.

High performance liquid chromatography; The home-made HPLC used with fluorometric detector has been previously described in detail[14]. TSK GEL-210 (Toyo Soda Co. Ltd., Tokyo) which was a 10 μm of particle size sulfonated porous polystyrene polymer was packed into a small metal tube (2×50 mm). Separation of catecholamines was carried out on this microcolumn as follows. Elution buffer (2.35 N citrate buffer pH 5.35 containing 5% MeOH) was delivered into the microcolumn at the flow rate of 0.3 ml/min under the pressure of 40 to 50 kg/cm^2 at 70°C, while OPA reagent, prepared according to the directions by Benson and Hare[2] (1975), was introduced as the post column manner at the flow rate of 0.5 ml/min into the mixing manifold. Thereafter the fluorescence intensity was measured by a model FLD-1 (Shimazu Co. Ltd., Tokyo) with EM-4 filter for emission.

Reproducibility of this analyticl method; For the purpose of testing the reproducibility of this analytical method, 20 μl of the standard mixture containing 150 picomoles of noradrenaline, dopamine and DHBA was injected into the column ten times and then measured the height and the area of each peak. The supernatant of the same rat brain homogenate was divided into several fractions, seven fractions of which were treated as described adove and used to measure the height of each peak for the reproducibility test.

RESULTS

Chromatographic separation of noradrenaline, dopamine and DHBA using the TSK GEL-210 microcolumn was shown in Fig. 2(a). While working curves for 20 μl of the standard mixture containing noradrenaline, dopamine and DHBA were linear at least to 150 picomoles as shown in Fig. 1. The peak height ratios of noradrenaline and dopamine to DHBA were constant.

The recovery of the standard mixture containing 150 picomoles of each amine by the clean up procedure with the alumina column was 77.8±4.0% for noradrenaline, 61.0±2.9% for dopamine and 72.8±2.9% for DHBA (mean± SD, n=10).

The reproduciblility of the HPLC procedure was tested for 20 μl of the standard mixture

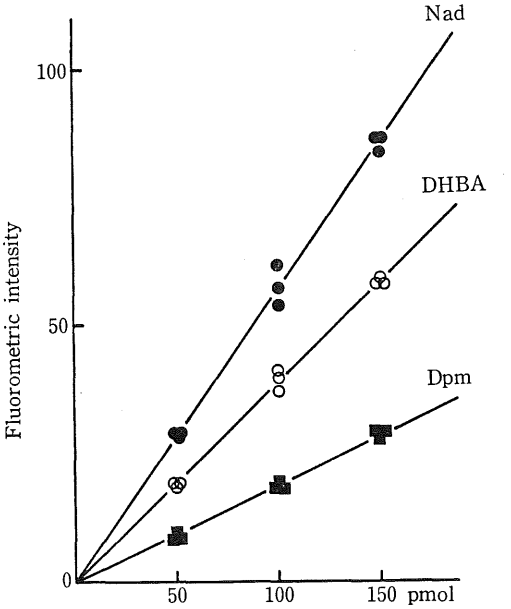

Fig. 1. Standard curves for noradrenaline (Nad), dopamine (Dpm) and 3, 4-dihydroxybenzylamine (DHBA) as internal standard.

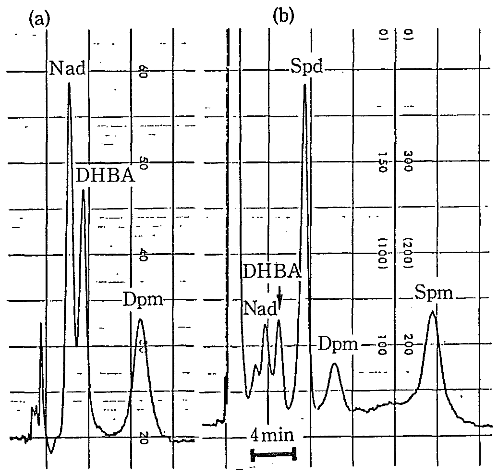

Fig. 2. (a) Chromatogram of OPA derivatives of 20 μl of standard mixture containing 150 pmol noradrenaline (Nad), dopamine (Dpm) and 3, 4-dihydroxybenzylamine (DHBA) as internal standard.
(b) Typical chromatogram of rat brain amines treated as described in the text. Spd: spermidine, Spm: spermine.

containing 150 picomoles of noradrenaline, dopamine and DHBA, and also for the aliquots of the supernatant of the rat brain homogenate. In the standard mixture, each peak height was 161.4±5.5 mm (3.4%) for noradrenaline, 53.5 ±2.3 mm (4.3%) for dopamine and 105.9±1.7 mm (1.6%) for DHBA [mean±SD, (precision of SD), n=10], while each peak area was 1197.1 ±34.4 mm^2 (2.9%), 854.8±26.1 mm^2 (3.1%) and 897.7±25.3 mm^2 (2.8%), respectively. Either peak height method or peak area method could be used, the former was used in this study from view points of simplicity.

To test the reproducibility of the total analytical method, the same supernatant of a rat brain was analyzed seven times. The content of noradrenaline and dopamine was 2.005± 0.086 nmol/g wet tissue (4.3%) and 4.184± 0.127 nmol/g wet tissue (3.0%) [mean±SD, (precision of SD)], respectively.

As regards the stability of catecholamines, when stored at −10°C, the TCA supernatant of the brain homogenate remained constant for a month, while the alumina column eluate was remained constant for at least a week. In addition, there was no difference in the contents of these amines in the supernatant obtained after centrifugation between at 1,500×g and 100,000×g for 15 min.

The average of each catecholamine content of five whole rat brains by the present method were 2.027±0.234 nmol/g wet tissue for noradrenaline and 4.333±0.365 nmol/g wet tissue for dopamine (mean±SD), which correspond to other chromatographic data[6,10].

DISCUSSION

Since the independent localization of the brain catecholamines has been proved with histochemical technique by several workers[8,12], it has been considered to be necessary to develop an available microanalysis of catecholamines in a small tissue. Further, it needs to analyze substances which relate to the catecholamine synthesis systematically. Recently, it has been possible to determine small amounts of catecholamines of biological samples in picomole range using the radioisotopic method[3,9] and HPLC combined with a high sensitive detector[7]. However, there is still a difficult problem for microestimation of biological amines.

In the present separation method, the sensitive and reproducible determination of noradrenaline and dopamine in a small animal brain using HPLC with the fluorometic OPA reagent was achieved. Moreover, the clean up technique presented here using the simple alumina column may be routinely utilized with easiness of operation and the good recovery and reproducibility.

In regard to the clean up procedure with the alumina method, distilled water[1,4] or Tris buffer[5,7,10] has been used to wash the column. However, the latter was not adequate in the present method, because Tris reacts with the OPA reagent. The washing of the alumina column with 0.2 M phosphte solution (pH 8.5) and the elution of catecholamines with 1 ml of 1 N acetic acid offered the recovery ratio of 60 to 80% of catecholamines with precision of ± 3 to $\pm 4\%$ SD and satisfactorily washed out other amines, especially spermidine and spermine as shown in Fig. 2(b). For the accurate microanalysis of catecholamines in the brain by this OPA method, it is necessary to wash out those polyamines which are contained in a large amount in the brain tissue and react with the OPA reagent. From view points of simplicity and reproducibility, when handling many samples, the alumina clean up procedure by column method was advantageous over conventional batch-wise method.

DHBA was a suitable reagent as an internal standard for noradrenaline and dopamine as described by other authors[5-7,10].

The variation coefficients of reproducibility were 4.3% for noradrenaline and 3.0% for dopamine in the rat brain. HPLC by sulfonated porous polystylene column was superior to the usual one by reversed-phase ODS column, in view of reproducibility of the separation and also of the life time of the column. For the detection of noradrenaline and dopamine, the postcolumn detection by OPA method was superior to usual detection method such as THI method or electrochemical detection (ECD) method; the THI method had disadvantages such as complicated reaction systems or poor sensitivity for dopamine and DHBA as the internal standard for catecholamines, and ECD method had a disadvantage of poor reproduiibility due to the change of characteristics of the glassy carbon electrode, though both methods were more sensitive and selective to noradrenaline than the present OPA method.

The detectable limit of this method was 10 picomoles for noradrenaline and 20 picomoles for dopamine which corresponded to about 1.7 ng and 3 ng, respectively. Only 10 to 20 mg wet weight of the brain tissue could be required for this method.

Since biological amines, noradrenaline and dopamine, may play some important roles which relate to other amines in an organism, it is necessary to develop a systematical analysis of these amines in the same sample. By the modification of the present OPA method, it may be possible to determine amino compounds in the same sample systematically.

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