Journal of Chromatography, 137 (1977) 357–362

CHROM. 9968

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF RAT-BRAIN DOPAMINE AND NOREPINEPHRINE

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(First received November 5th, 1976; revised manuscript received January 18th, 1977)

SUMMARY

A high-performance liquid chromatographic method for the assay of dopamine (DM) and norepinephrine (NE) in rat brain has been developed. 3,4-Dihydroxybenzylamine was added to the sample as an internal standard. The isolated amines and the internal standard were subjected to reaction with fluorescamine, separated liquid chromatographically on a glycol-type gel and determined fluorimetrically. The standard deviation of the method was $\pm 2.7\%$ for DM and $\pm 5.3\%$ for NE. The contents of DM and NE in rat brain, with or without administration of a tyrosine hydroxylase inhibitor, as measured by the proposed method were always lower than those obtained by the trihydroxyindole method.

INTRODUCTION

It has been reported[1] that fluorescamine reacts with dopamine (DM) and norepinephrine (NE) to give highly fluorescent products, which can be separately detected by high-performance liquid chromatography (HPLC). The method was applied to the determination of DM and NE in rat brain with the aid of preliminary isolation by alumina adsorption. However, careful sample pre-treatment was necessary in order to obtain reproducible results.

In this work, in order to simplify the pre-treatment procedure, 3,4-dihydroxybenzylamine (DHBA)[2] was utilized as an internal standard, added to the sample at the beginning of the isolation procedure. Further, the polystyrene gel column[1] was replaced with a glycol-type gel column in order to separate the fluorescamine derivatives of DM, NE and DHBA completely. The amounts of DM and NE in rat brain obtained by the method proposed here were compared with those obtained by the trihydroxyindole (THI) method[3].

EXPERIMENTAL

Materials

Alumina (Woelm neutral) was purified according to Anton and Sayre[3]. Dopamine hydrochloride and norepinephrine were obtained from Nakarai Chemicals Co.

(Kyoto, Japan). Fluorescamine was obtained from Hoffmann-La Roche (Nutley, N.J., U.S.A.).

3,4-Dihydroxybenzylamine hydrobromide (m.p. 183–186°) was synthesized according to the procedure of Refshauge *et al.*[2], which can be summarized as follows. Twenty millilitres of a 25% solution of hydrogen bromide in acetic acid (Nakarai Chemicals) containing 1 g of 4-hydroxy-3-methoxybenzylamine hydrochloride (Aldrich, Milwaukee, Wisc., U.S.A.) was refluxed at 140° for 5 h. After standing at room temperature overnight, colourless needles (1.1 g) were obtained. Analysis: calculated for $C_7H_9NO_2 \cdot HBr$, C 38.20, H 4.58, N 6.37 and Br 36.31%; found, C 38.49, H 4.61, N 6.47 and Br 36.20%.

All other chemicals were of reagent grade.

Administration of α-MT

dl-α-Methyl-*p*-tyrosine methyl ester (α-MT, Sigma, St. Louis, Mo., U.S.A.) in saline solution was administered to rat intraperitoneally with a dose of 250 mg/kg[4]. Four hours after injection, the rat was killed and the brain treated as described below.

Preparation of rat-brain samples

Comparison between the proposed method and the THI method. The brains of male Wistar rats (7 weeks of age) were each weighed (W), homogenized in 8 ml of 0.4 N perchloric acid and the homogenate was centrifuged at 10,000 g for 20 min. The supernatant was weighed (T) and divided into two approximately equal portions. Each portion was weighed (A and B), the former portion being used in the proposed method and the latter in the THI method. The weights of brain used for the determinations were $W \cdot A/T$ in the proposed method and $W \cdot B/T$ in the THI method.

Reproducibility test. Ten rat brains (16.9 g, rats 12 weeks of age) were suspended in 85 ml of 0.4 N perchloric acid, homogenized and centrifuged as described above. The supernatant (82 ml) was divided to 4-ml portions, seven of them being used for the reproducibility test with the proposed method.

Preparation of working curves. One millilitre each of the perchloric acid solution of the following amounts of DM and NE were added to each of the supernatants described under *Reproducibility test*: DM, 0.261, 0.522 and 0.783 μg/ml; NE, 0.159, 0.137 and 0.476 μg/ml. The weights of the added amines were plotted against the peak height ratios of the amines to the internal standard (1.23 μg of DHBA as HBr salt); the blank value of the ratio was subtracted.

Alumina adsorption of the amines in the supernatant

To each supernatant were added 0.1 ml of DHBA·HBr (1.23 μg) in 0.1 M sodium borate buffer solution (pH 8.5) containing 10 μg/ml of EDTA and 20 μg/ml of ascorbic acid, 1 ml of 0.2 M EDTA solution, 4 N ammonia solution to give a pH of about 8.5 and 0.4 g of alumina. The suspension was stirred with a glass rod for 5 min and the supernatant was aspirated and discarded. The alumina was transferred into a glass column and the adsorbed amines were eluted with 2 ml of 0.4 N acetic acid in methanol in the proposed method and with 6 ml of 0.05 N perchloric acid in the THI method. The eluate was evaporated under reduced pressure at 30° and dried over silica gel in a desiccator.

High-performance liquid chromatography of DM and NE in rat brain

The residue obtained after evaporation of the eluent from the alumina column was dissolved in 70 μl of 0.1 *M* sodium borate buffer (pH 8.5) containing 10 μg/ml of EDTA and 20 μg/ml of ascorbic acid and 30 μl of a 0.02% solution of fluorescamine in acetone were immediately added. Then 20–40 μl of the solution were directly applied on to the column, some of the solution being stored in a refrigerator in order to test its stability.

TSK gel 160 (Toyo Soda Manufacturing Co., Tokyo, Japan), particle size 10 μm was used as the column packing. A Minimicro Model WU-1 high-speed piston pump (Kyowa Seimitsu Co., Tokyo, Japan) delivered mobile phase at a rate of 0.44 ml/min (pressure 88–93 kg/cm^2) to a column in a thick-walled glass tube (500 × 3 mm) fitted with a head for syringe injection through a rubber septum. While the injection was being performed, the flow of mobile phase was stopped. The eluted fluorophores were measured with an Aminco Fluorocolorimeter No. J4-743 equipped with an ultraviolet lamp (No. JA-7125, maximal energy at 360 nm), a Corning No. JJ-7113 primary filter, a secondary filter (No. JA-7116) and a quartz flow-through cell. The intensities were recorded on a Technicorder, Type 3047 (Yokogawa Electric Works, Tokyo, Japan). All lines were connected with PTFE tubing (0.5 mm I.D., 2 mm O.D.). HPLC was performed with a mixture of methanol and 0.5 *M* Tris–hydrochloric acid buffer (pH 8.0) containing 10 μg/ml of EDTA and 20 μg/ml of ascorbic acid.

THI method

Almost the same procedure as described by Anton and Sayre[3] was performed.

For NE. A 1-ml aliquot of the perchloric acid eluate was neutralized with 0.3 ml of 0.13 *N* sodium hydroxide solution. To the solution were added 0.2 ml of 0.5 *M* phospahte buffer (pH 7.0) and 0.05 ml of 0.14% zinc sulphate solution, and then 0.05 ml of 0.12% potassium hexacyanoferrate(III) for oxidation, with cooling in an ice-bath. After 15 min, 1 ml of a freshly prepared 0.2% solution of ascorbic acid in 10 *N* sodium hydroxide solution was added. After standing for 15 min at room temperature, the fluorescence intensity of the solution was measured at 510 nm with excitation at 400 nm. A tissue blank value was obtained without the hexacyanoferrate(III) oxidation step. A working curve was obtained by addition of standard NE to the pooled sample as described above.

For DM. To a mixture of a 0.2-ml aliquot of the perchloric acid eluate, 0.01 ml of 70% ethanol and 0.1 ml of 0.5 *M* phosphate buffer (pH 7.0) was added 0.02 ml of 0.5% sodium periodate solution. Just after 1 min, 0.1 ml of a freshly prepared 2.5% solution of sodium sulphite in 4.5 *N* sodium hydroxide solution was added, followed 20 sec later by 0.55 ml of a mixture of distilled water, 0.5 *M* citrate buffer (pH 4.0) and 3 *M* orthophosphoric acid (28:10:17). The fluorescence intensity was measured at 370 nm with excitation at 325 nm. A tissue blank value was obtained without the sodium periodate oxidation step. A working curve was obtained by addition of standard DM to the pooled sample as described above.

RESULTS

DHBA could not be separated from DM on Hitachi 3010 or another polystyrene gel of the same type (TSK 110; Toyo Soda), while the glycol-type gel TSK 160

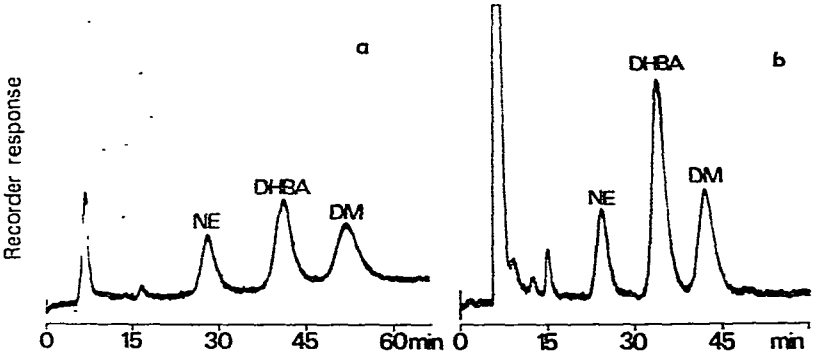

Fig. 1. (a) Chromatogram of fluorescamine derivatives of authentic dopamine (DM), norepinephrine (NE) and 3,4-dihydroxybenzylamine (DHBA) (internal standard). Buffer solution (70 μl) containing NE (0.404 μg), DM (0.690 μg) and DHBA (0.861 μg) was mixed with 30 μl of 0.02% fluorescamine in acetone and 10 μl of the reaction mixture were subjected to HPLC. Eluting solvent, 0.5 *M* Tris–hydrochloric acid buffer (pH 8.0)–methanol (75:25, v/v); temperature, 52°; pressure, 88–93 kg/cm^2. (b) Chromatogram of dopamine and norepinephrine obtained from half a rat brain, treated as described in the text. HPLC conditions as in (a) except the eluting solvent was 0.5 *M* Tris–hydrochloric acid buffer (pH 8.0)–methanol (70:30, v/v).

used in this work separated NE, DHBA and DM, as shown in Fig. 1a. The best separation was achieved when the volume ratio of methanol to 0.5 *M* Tris–hydrochloric acid buffer (pH 8.0) in the eluting solvent was 25:75 and the flow-rate was 0.48 ml/min at 52°. However, in order to reduce the time required for analysis, the ratio 30:70 was subsequently used. The order of elution from the column was the same as that from the cation-exchange resin Zipax SCX with 0.1 *N* perchloric acid as an eluting solvent[2], namely NE, DHBA and DM.

The chromatogram obtained by the treatment of half a rat brain sample is shown in Fig. 1b.

The peak-height ratio of the amines to DHBA remained constant for at least 3 days when the fluorescamine derivatives of a brain sample were stored in a refrigerator. The peak-height ratio of DM or NE to DHBA, obtained after the addition of

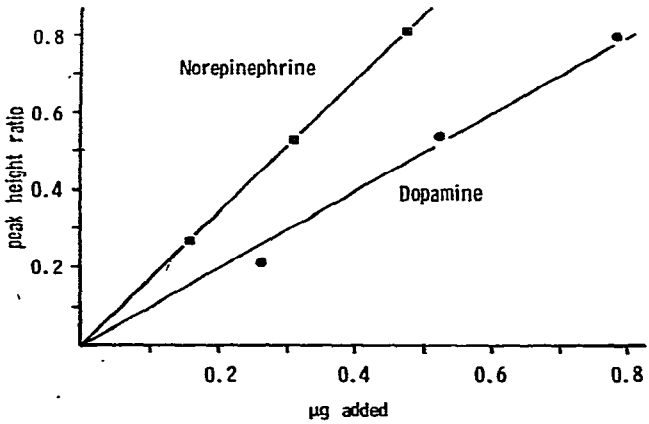

Fig. 2. Working curves for dopamine and norepinephrine with 3,4-dihydroxybenzylamine (1.23 μg as HBr salt) as the internal standard. Blank values were subtracted from the peak-height ratios.

known amounts of standard amines as described under Experimental, was proportional to the amounts present, as shown in Fig. 2.

The reproducibility of the method was tested with pooled rat-brains (12 weeks of age) homogenate. The amount of DM was found to be 0.685 ± 0.019 (S.D., $n = 7$) μg/g and of NE 0.300 ± 0.016 μg/g.

The contents of the amines in rat brain (7 weeks of age) obtained by the proposed method were compared with those obtained by the THI method. The proposed method gave values of 0.591 ± 0.043 (S.D., $n = 7$) μg/g for DM and 0.256 ± 0.009 (S.D., $n = 7$) μg/g for NE, while the THI method gave values of 0.660 ± 0.021 (S.D., $n = 7$) μg/g for DM and 0.305 ± 0.020 (S.D., $n = 7$) μg/g for NE, *i.e.*, the proposed method gave lower values. The same trend was also observed when α-methyl-*p*-tyrosine methyl ester, an inhibitor of tyrosine hydroxylase, was administered to rats intraperitonealy: the proposed method have values of 0.167 ± 0.034 (S.D., $n = 7$) μg/g for DM and 0.113 ± 0.016 (S.D., $n = 7$) μg/g for NE, and the THI method 0.209 ± 0.043 (S.D., $n = 7$) μg/g for DM and 0.166 ± 0.011 (S.D., $n = 7$) μg/g for NE.

DISCUSSION

DHBA was originally used by Refshauge *et al.*[2] as an internal standard in the HPLC separation and electrochemical determination of animal-brain catecholamines. As it has the same catechol moiety in the molecule to be selectively adsorbed on alumina and the same primary amino group to react with fluorescamine, it was also useful as an internal standard in the method proposed here. Noradrenalone was another possible internal standard as it has the same retention volume as DHBA on the gel, but it was not easy to obtain pure standard material.

As the internal standard is added at the beginning of the isolation procedure, one can treat the sample without concern about losses of material during the process. In a preliminary experiment only a negligible loss was observed in the deproteinization step. Further, for reproducible results (the coefficient of variation was 2.7% for DM and 5.3% for NE), there is no need to be concerned about oxidation during the pre-treatment of the amines, so that the solutions and eluting solvent used need not be deaerated[2].

The limit of determination of the proposed fluorometric procedure is about 100 ng of the amines, while with an electrochemical detector method[2] and the ethylenediamine condensation method[5] down to about 10 ng can be determined. The enzyme radiochemical method[6] can be used to determine DM at the nanogram level. This difference should be reduced by the use of a more elaborate column and detector.

In the proposed method, epinephrine (a secondary amine) does not react with fluorescamine and is not detected by fluorimetry, although it has been identified in rat-brain tissue by mass fragmentography[7]. On the other hand, in the ion-exchange chromatography of catecholamines[2], epinephrine was reported to be eluted at the same retention volume as DHBA[8], and care should therefore be taken in the analysis of rat-brain catecholamines.

The lower values obtained by the proposed method compared with those obtained by the THI method should be ascribed to the fact that in the former instance DM and NE were determined separately and mutual contamination was avoided.

In a preliminary experiment, we found that the present method was applicable to the determination of DM and NE in urine with direct adsorption on alumina and no further pre-treatment.

ACKNOWLEDGEMENTS

The authors express their thanks to Drs. Y. Suganuma and H. Tani of Kowa Co. for their interest and valuable discussions. Thanks are also due to Mrs. H. Saito and K. Sakane of Toyo Soda Manufacturing Co. for kindly supplying the TSK gel.

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