Journal of Chromatography, 311 (1984) 390—395
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2278

Note

Assay for histidine decarboxylase in rat stomach and brain by high-performance liquid chromatography with fluorescence detection

YUKITAKA NAKANO, MASATOSHI YAMAGUCHI*, YASUTO TSURUTA** and YOSUKE OHKURA*

Faculty of Pharmaceutical Sciences, Kyushu University 62, Maidashi, Higashi-ku, Fukuoka 812 (Japan)

and

TOSHINOBU AOYAMA and MASAYOSHI HORIOKA

Hospital Pharmacy, Kyushu University 71, Maidashi, Higashi-ku, Fukuoka 812 (Japan)

(First received April 25th, 1984; revised manuscript received June 13th, 1984)

Histidine decarboxylase (HDC, EC 4.1.1.22, L-histidine carboxylase) plays an important role in the formation of histamine in tissues, and it catalyses the decarboxylation of histidine to histamine in the presence of pyridoxal-5'-phosphate (PALP). The enzyme is found in many tissues such as fetal rat liver [1], mouse mastocytoma [2], mammalian brain [3], rat stomach [4] and hamster placenta [5]. Although the activity in these tissues is usually very low, its assay has been required for biological studies on the biosynthesis, storage and release of histamine.

The activity has been assayed by radiochemical and conventional fluorimetric methods. Although the radiochemical methods [6—12] are highly sensitive, they are rather complicated and require expensive substrates. The fluorimetric methods [13, 14], based on the reaction of enzymatically formed and endogenous histamine with *o*-phthalaldehyde (OPA) in alkaline medium to produce a fluorescent product, permit the assay of the activity in partially purified enzyme preparations but they are not very well suited for the assay in crude en-

*Present address: Faculty of Pharmaceutical Sciences, Fukuoka University, Nanakuma, Jonan-ku, Fukuoka 814-01, Japan.
**Present address: Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University, Sanzo, Higashimuramachi, Fukuyama 729-02, Japan.

0378-4347/84/$03.00

zyme preparations which are rich in various biogenic amines. The methods are insensitive and so require relatively large amounts of biological samples.

We have previously presented a selective and sensitive method for the assay of histamine in urine and plasma by reversed-phase high-performance liquid chromatography (HPLC) with fluorescence detection, based on the precolumn derivatization of the amine with OPA [15, 16].

This present paper describes an HPLC method for the assay of HDC in biological materials. Histamine formed from the substrate histidine under the optimum conditions for the enzyme reaction is separated from unreacted histidine by ion-exchange chromatography and then determined by the HPLC method. The enzyme preparations from rat stomach and brain were employed as those with relatively high and extremely low activities of HDC, respectively, to establish the assay procedure.

EXPERIMENTAL

Materials and methods

All chemicals were of reagent grade, unless otherwise noted. Deionized and distilled water was used. Amberlite CG-50 (Na^+; 200—400 mesh; Rohm and Haas, Philadelphia, PA, U.S.A.) (50 g) was washed successively with 500 ml of 2 *M* sodium hydroxide (three times), 500 ml of water (three times), 500 ml of 2 *M* hydrochloric acid (three times) and 500 ml of water (three times), and finally equilibrated with 0.2 *M* phosphate buffer (pH 6.5). The amberlite CG-50 column was prepared by packing 0.25 ml of the resin in a glass tube (100 × 4 mm I.D.). An HPLC column (150 × 4 mm I.D.) of TSK-GEL LS-410 ODS SIL (particle size 5 μm; Toyo Soda, Tokyo, Japan) [16] was used. The column can be used for more than 1000 injections when washed with aqueous methanol (1 : 1, v/v) at a flow-rate of 0.5 ml/min for ca. 30 min after every analyses.

HDC preparations from rat stomach and brain

Rat stomach and brain were obtained from male Wistar rats (5—6 weeks of age; weight 160—200 g) and male Sprague—Dawley rats (6—7 weeks of age; weight 250—300 g), respectively. The rats were killed by a blow on the neck and subsequently decapitated. The stomach was cut open along the greater curvature and the mucosal surface was cleaned thoroughly with ice-cold saline. The corpus ventriculi area of the stomach was scraped off and homogenized in 8 vols. of 0.1 *M* acetate buffer (pH 5.5) containing 0.2 m*M* dithiothreitol and 1.0% (v/v) polyethyleneglycol (average molecular weight, 300). The homogenate was centrifuged at 10,000 *g* for 30 min in a refrigerated centrifuge and the supernatant was used as the HDC preparation. The whole rat brain was rapidly removed, blotted and homogenized with 20 ml of the acetate buffer in the same way. The homogenate was centrifuged at 10,000 *g* for 20 min. The supernatant was used for the assay. The whole procedure was carried out at 0—5°C. The protein concentration in both enzyme preparations was adjusted to ca. 100 μg per 0.1 ml with the acetate buffer. The protein concentration was measured by the method of Lowry et al. [17] using bovine serum albumin as a standard protein.

Apparatus

A Shimadzu LC-3A high-performance liquid chromatograph was used, equipped with a Rheodyne 7125 syringe-loading sample injector valve (100-μl loop) and a Shimadzu RF-530 fluorescence spectromonitor fitted with a 12-μl flow-cell operating at an emission wavelength of 450 nm and an excitation wavelength of 360 nm. The sensitivity of the spectromonitor was set in general at the ranges 2 and 256 of the high level in the assay for HDC in stomach and brain, respectively. Uncorrected fluorescence excitation and emission spectra were measured with a Hitachi MPF-4 spectrofluorimeter in quartz cells (optical path-length, 10 × 10 mm); spectral band-widths of 5 nm were used in both the excitation and emission monochromators.

Procedure

Cofactor solution was 0.2 *M* phosphate buffer (pH 6.8) containing 12.5 μM PALP for the assay of HDC in stomach or 37.5 μM for the assay in brain, 250 μM dithiothreitol, 1.25% (v/v) polyethyleneglycol and 12.5 μM aminoguanidine. To 0.8 ml of the solution was added 0.1 ml of the HDC preparation. The mixture was preincubated at 37°C for 10 min and then incubated again at 37°C for 60 min for HDC assay in stomach and for 180 min for the assay in brain, after addition of 0.1 ml of 25 m*M* histidine (substrate solution). The reaction was terminated by adding 0.2 ml of 2.4 *M* perchloric acid. The mixture was centrifuged at 7000 *g* for 5 min. To the supernatant (1.0 ml) were added 0.2 ml of 1.8 *M* potassium hydroxide and 1.0 ml of 0.5 *M* phosphate buffer (pH 6.5). The mixture was placed in an ice-bath for ca. 10 min, and then centrifuged at 1500 *g* for 10 min. The supernatant (2.0 ml) was poured on to an Amberlite CG-50 column. Histidine in the column was washed twice with 1.0 ml of 0.2 *M* phosphate buffer (pH 7.0) and then twice with 1.0 ml of water. The adsorbed histamine was eluted with 1.0 ml of 0.4 *M* sodium hydroxide. To the elute, 50 μl of 74.6 m*M* OPA in methanol were added and the resulting mixture was allowed to stand at 25°C for exactly 3 min to develop the fluorescence. Sulphuric acid (1.5 *M*, 100 μl) was added to stop the reaction. The reaction mixture (100 μl) was subjected to HPLC. The mobile phase was a mixture of 0.2 *M* sodium chloride, methanol and 0.1 *M* hydrochloric acid (55 : 60 : 1.25, v/v/v). The flow-rate was 0.5 ml/min. The column temperature was ambient (20—25°C).

For the blank, the same procedure was carried out except that the order of addition of the substrate solution and the perchloric acid solution was reversed. The net peak height in the chromatogram was used for the quantification of histamine. The amount of histamine was calibrated by means of the standard addition method: the substrate solution (0.1 ml) in the procedure for the blank was replaced with histamine standard solution (500 and 17.5 pmol per 0.1 ml for rat stomach and brain, respectively) dissolved in the substrate solution.

RESULTS AND DISCUSSION

The conditions of the fluorescence reaction and HPLC were almost the same as described previously [15, 16].

Fig. 1 shows typical chromatograms obtained with the brain HDC preparation prepared according to the procedure. The peak due to histamine was ob-

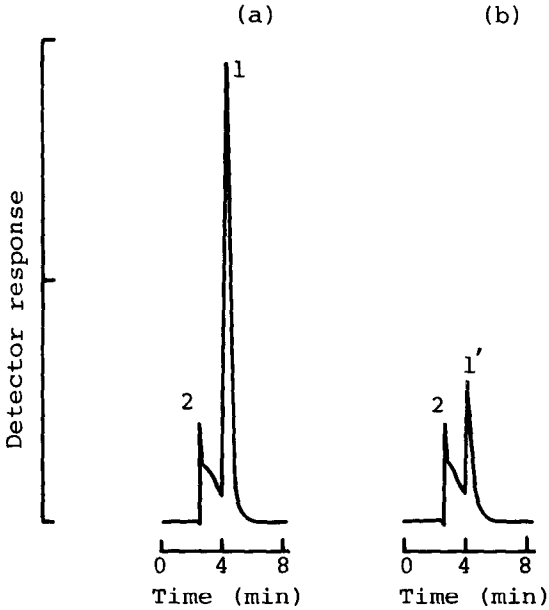

Fig. 1. Chromatograms obtained with (a) the HDC preparation from rat brain and (b) the blank carried through the procedure (see text). Peaks: 1 = histamine formed enzymatically plus endogenous histamine; 1' = endogenous histamine; 2 = biogenic amines other than histamine. The activity of HDC was 0.79 pmol per min per mg of protein. The HDC preparation used contains 109 μg of protein and 5.5 pmol of endogenous histamine in 0.1 ml.

served at the retention time of 4.5 min in each of the chromatograms (peaks 1 and 1'). The eluates from peaks 1 and 1' have the same fluorescence excitation (maximum 360 nm) and emission (maximum 450 nm) spectra. Peak 1' in the chromatogram of the blank increases in height in proportion to increasing amount of protein in the HDC preparation, and is due to the endogenous histamine in the preparation. Peak 2 increases in height with increasing amount of protein in the preparation, and is ascribable to biogenic amines other than histamine.

The patterns of chromatograms obtained with the HDC preparation from stomach were virtually identical with those of Fig. 1. However, the levels of both the enzyme activity and endogenous histamine in stomach are very much greater than in brain, and, therefore, compared to the peak for histamine, the peak for biogenic amine (corresponding to peak 2 in Fig. 1) was relatively very small.

Since the substrate histidine forms a fluorophore which shows similar chromatographic and fluorescence spectral behaviours to that of histamine when it reacts with OPA (structures of the fluorophores of histamine and histidine remain unknown), adequate separation of histamine from unreacted histidine is required before derivatization of histamine with OPA. This was successfully carried out by chromatography on a small column of Amberlite CG-50. The recovery of 1.0 nmol of histamine in the presence of 1.0 μmol of histidine was 98.2 ± 2.4% [mean ± standard deviation (S.D.), $n = 15$].

A maximum and constant HDC activity was obtained in the presence of 2.0—5.0 m*M* histidine in the incubation mixture with an observed Michaelis

constant of 0.40 m*M*; 2.5 m*M* was used as a saturating concentration for the enzyme reaction. HDC was most active at pH 6.8 in the presence of 2.5 m*M* histidine in the incubation mixture. Maximum activity was attained in phosphate buffer at concentrations of 0.15—0.3 *M* in the incubation mixture; 0.2 *M* phosphate buffer (pH 6.8) in the cofactor solution was employed. PALP in the incubation mixture provided a maximum activity in the concentration ranges of 5—20 μM and 20—40 μM for the assay of HDC in stomach and brain, respectively; 10 and 30 μM respectively, were selected for the standard procedure. The prescribed concentrations of dithiothreitol and polyethyleneglycol, stabilizers of HDC, in the incubation mixtures were optimal [14].

Histaminase present in the enzyme preparation catalyses the conversion of histamine to imidazoleacetaldehyde and thus a histaminase inhibitor aminoguanidine is used in the HDC assay procedure [14].

Aminoguanidine in the incubation mixture gave a maximum activity of HDC at concentrations of 8—20 μM, 10 μM was used in the procedure. Histamine is also converted to N^{τ}-methylhistamine by histamine N-methyltransferase-mediated reaction. The effect of N-methyltransferase on the amount of histamine formed was examined using 10 μM histamine solution in place of the substrate solution in the procedure. The amount of histamine did not vary with or without incubation for 180 min. Therefore, it seemed that N-methyltransferase does not affect the assay of HDC.

The enzyme activity was linear with time up to at least 3 and 6 h for the enzyme preparations from stomach and brain, respectively, when incubated at 37°C. The amounts of histamine formed for the prescribed incubation times were proportional to the amount of protein in the HDC preparations (0.1 ml) up to 1.5 mg. The HDC preparations were adjusted to contain ca. 100 μg of protein per 0.1 ml, respectively, in the procedure.

A linear relationship was obtained between the peak height of histamine and the amount of the amine added in the range of 2.0 pmol to 5.0 nmol to the enzyme reaction mixture. The recoveries of histamine added to the reaction mixtures of the blanks in HDC assay of stomach and brain in the amounts of 500 and 10 pmol were 93.2 ± 3.0% and 95.3 ± 2.4% (mean ± S.D., n = 12 in each case), respectively.

The lower limits of detection for histamine formed enzymatically were 110 pmol per assay tube in stomach HDC assay and 1 pmol per assay tube in brain HDC assay. The limit was defined as the amount giving 1.2 times the height of the peak in the blank for the reason that the amounts of enzymatically formed and endogenous histamine can be measured fairly precisely (coefficient of variation below 3%).

HDC activities in the preparations from rat (male, Wistar, 5—6 weeks of age) stomach and rat (male, Sprague—Dawley, 6—7 weeks of age) brain were 80.5 ± 9.1 and 0.79 ± 0.15 pmol per min per mg of protein, respectively (mean ± S.D., n = 10 in each case). The values were similar to those obtained by other workers [6—14].

This study provides the first HPLC method with fluorescence detection for the assay of HDC. The method is highly sensitive and so requires only 100 μg of protein from rat stomach or brain. The method may permit the assay of HDC in preparations from other tissues. Therefore, it should be useful for biological investigations of HDC in place of radiochemical methods.

REFERENCES

1 R. Håkansaon, Biochem. Pharmacol., 12 (1963) 1289.
2 D. Aures and W.W. Nall, Fed. Proc., Fed. Amer. Soc. Exp. Biol., 25 (1966) 691.
3 J.C. Schwartz, G. Lampart and C. Rose, J. Neurochem., 17 (1970) 1527.
4 R. Håkanson and C. Owman, Biochem. Pharmacol., 15 (1966) 489.
5 R. Håkanson, Eur. J. Pharmacol., 1 (1967) 42.
6 Y. Kobayashi, Anal. Biochem., 5 (1963) 284.
7 D. Aures and W.G. Clark, Anal. Biochem., 9 (1964) 35.
8 R.J. Levine and D.E. Watts, Biochem. Pharmacol., 15 (1966) 841.
9 M.A. Beaven, Anal. Biochem., 84 (1978) 638.
10 G. Kahlson and E. Rosengren, Physiol. Rev., 48 (1968) 155.
11 M.A. Reilly and R.W. Schayer, Brit. J. Pharmacol., 34 (1968) 551.
12 M. Baudry, M.P. Martres and J.C. Schwarts, J. Neurochem., 21 (1973) 1301.
13 K.M. Taylor and S.H. Snyder, J. Neurochem., 19 (1972) 1343.
14 T. Watanabe, H. Nakamura and H. Wada, Biochem. Pharmacol., 28 (1979) 1149.
15 Y. Tsuruta, K. Kohashi and Y. Ohkura, J. Chromatogr., 146 (1978) 490.
16 Y. Ohkura, K. Kohashi, Y. Tsuruta and S. Ishida, Advan. Biosci., 33 (1982) 1289.
17 O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem., 193 (1951) 265.