Journal of Chromatography, 221 (1980) 155—160
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 652

Note

Gas chromatographic determination of monoamine oxidase activity using mixed substrates

AKIKO UJI and SATOSHI KAWAI*

Gifu College of Pharmacy, Mitahora, Gifu (Japan)

and

TOSHIHARU NAGATSU

Laboratory of Cell Physiology, Department of Life Chemistry, Graduate School, Tokyo Institute of Technology, Nagatsuda, Midori-ku, Yokohama (Japan)

(Received April 1st, 1980)

Monoamine oxidase (MAO) catalyzes the oxidative deamination of monoamines. A mitochondrial preparation has been reported to contain two forms of MAO with different specificities: form-A and form-B, these forms may be distinguished by their differences in sensitivity to inhibitors and substrate specificity [1].

Mixed substrate experiments have been carried out with the mitochondrial MAO from rat liver [2], human brain [3] and rat brain [4] in order to study substrate specificity and amine competition for MAO. However, we are not aware of any report dealing with the simultaneous determination of resulting products in mixed substrate experiments for the determination of MAO activity. The purpose of using a mixed substrates method in the present paper is to establish a useful method for estimating effects on MAO caused by various diseases as a change of MAO activity which is difficult to distinguish by the use of a single substrate. Various methods have been used for the assay of MAO activity: oxygen electrode [2, 5], spectrophotometry [6—8], fluorometry [9—14], radioisotopic assay [4, 15—20] and gas chromatography (GC) [21]. Reports dealing with GC for measuring enzymatic activity are not numerous, but a GC method generally makes it possible to study enzyme reactions with

0378-4347/80/0000—0000/$02.25

mixed substrates, offering an advantageous technique for the simultaneous determination of resulting products. A comparison of the enzyme reaction patterns obtained from mixed substrates may be applicable to clinical diagnosis, without an absolute determination of individual products. In the present study, mixed substrate experiments have been carried out with rat liver mitochondrial MAO. MAO activity has been determined by GC with benzylamine and β-phenylethylamine as the substrates. The products, benzaldehyde and phenylacetaldehyde were converted to their pentafluorophenylhydrazones and detected with an electron-capture detector.

EXPERIMENTAL

Materials

Reagents. Pentafluorophenylhydrazine (PFPH) was purchased from Aldrich (Milwaukee, WI, U.S.A.); benzylamine hydrochloride and β-phenylethylamine hydrochloride from Tokyo Kasei (Tokyo, Japan); benzaldehyde from Yoneyama Yakuhin (Osaka, Japan); phenylacetaldehyde from Wako Junyaku (Osaka, Japan), respectively. The aldehydes were distilled before use and kept under nitrogen.

Substrate solutions. Benzylamine (20 μmoles/ml) and β-phenylethylamine (10 μmoles/ml) solutions were prepared in the buffer solution.

Buffer solution. A 50 m*M* potassium phosphate solution, pH 7.4 was used.

Internal standard (IS) solution. An aldrin solution (0.25 μg/ml) was used in *n*-hexane.

Enzyme. Male Wister strain rats weighing 150—200 g were decapitated and their livers removed and homogenized in 6 volumes of 50 m*M* potassium phosphate buffer (pH 7.4). The mitochondrial fraction was prepared by differential centrifugation using the method of Hogeboom et al. [22]. The mitochondria were washed once by resuspending them in 50 m*M* potassium phosphate buffer and used as the enzyme preparation. This preparation was divided into small volumes and kept frozen until use. The enzyme protein content was estimated by the method of Lowly et al. [23] and was in the range of 5—10 mg/ml.

Apparatus and conditions

A Shimadzu GC-4APE gas chromatograph equipped with a 10-mCi ^{63}Ni electron-capture detector (ECD) was used. The GC conditions were as follows: a 2 m × 3 mm I.D. glass column packed with 3% XE-60 on 80—100 mesh Celite 545 (AW DMCS): column temperature 190°C; detector temperature 200°C and chart speed 0.25 cm/min.

Procedure for the assay of MAO activity

The incubation medium contained 1 μmole of benzylamine hydrochloride and 0.5 μmole of β-phenylethylamine hydrochloride in a total volume of 0.15 ml of 50 m*M* phosphate buffer, pH 7.4. The mixture was pre-incubated at 37°C for 5 min in a 10-ml centrifuge tube, after which the reaction was started by adding 0.1 ml of the mitochondrial preparation. After shaking for 30 min in an incubator at 37°C, the reaction was stopped by adding 0.01 ml of 60%

perchloric acid. *n*-Hexane (4 ml) containing 1 μg of aldrin as IS, was added to the reaction solution, followed by saturating with sodium chloride and the resulting aldehydes were extracted by mixing the solution on a homomixer. To 1 ml of the *n*-hexane extract in another tube, 0.1 ml of PFPH solution (3 mg/ml, 10% acetic acid in ethanol) was added and the mixture was allowed to stand for 1 h at room temperature. The reaction solution was washed with 1 ml of 6 *N* hydrochloric acid to remove any excess of PFPH, diluted five-fold with *n*-hexane and an aliquot of the solution was applied onto the GC column. A series of blanks was prepared by incubating the substrates in the absence of the mitochondrial preparation. The enzyme source was added immediately, followed by perchloric acid treatment, prior to extraction.

RESULTS AND DISCUSSION

The only report dealing with GC of MAO activity is that by Farris et al. [21], who reported a microassay for the determination of MAO activity using ECD, where *m*-iodobenzylamine was employed as a substrate and the oxidation product, *m*-iodobenzaldehyde was extracted into cyclohexane and measured by electron-capture GC. This technique was successfully applied to a sensitive and specific assay for determining MAO activity in serum and platelets. The selection of *m*-iodobenzylamine as a substrate for their study was based on three important considerations. First, the aldehyde formed from the oxidative deamination of the amine was quite stable to further oxidation; secondly, *m*-iodobenzylamine was a very good substrate for form-B MAO and finally, *m*-iodobenzaldehyde was highly sensitive to detection by ECD. However, their method suffers from the disadvantage of being limited to the use of a halogen-containing substrate, so a procedure of derivatizing the aldehydes formed to halogen-containing compounds which are very sensitive to ECD is general with regard to the possibility of using various substrates. On the other hand, it appears that most of the substrates which are attacked by MAO are of the β-phenylethylamine type. In this study, benzylamine and β-phenylethylamine were chosen as one of the simplest combinations of two substrates.

3% XE-60 was chosen as a suitable column packing for the GC separation, because it gave sharp symmetrical peaks and good separations. Aldrin was used as the internal standard. Fig. 1A shows a gas chromatogram of a standard solution containing IS.

A condensation reaction of PFPH and aldehydes in aqueous solution is known to be an effective derivatization process [24], which gives volatile, ECD-sensitive derivatives, extractable with organic solvents. The condensation reaction proceeded more readily in neutral media, but in the enzyme reaction solution, strong acidity derived by adding 0.01 ml of 60% perchloric acid and co-existing enzyme protein seemed to remarkably disturb the condensation reaction. A series of preliminary investigations was carried out in order to find suitable conditons for the extraction of aldehydes and the reaction with PFPH. *n*-Hexane was chosen as the solvent for extraction. Salting-out improved the extraction yield. The reagent concentration was about 60 times greater than that of the aldehydes and the reaction period was fixed at 1 h at room temperature in order to obtain constant reaction yields. PFPH was used as a

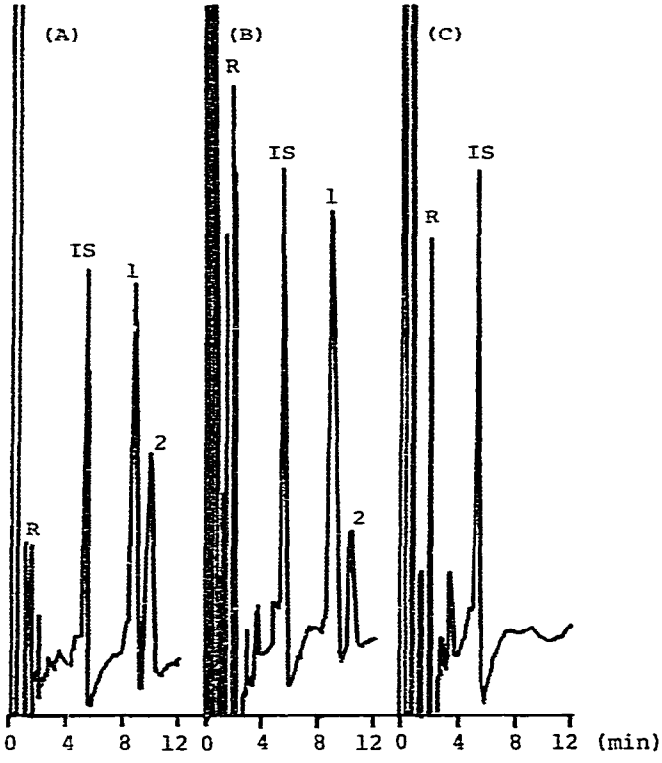

Fig. 1. Chromatograms obtained from 1-μl injections of (A) 47 pg/μl benzaldehyde and 150 pg/μl phenylacetaldehyde standard, (B) the product of a 30-min incubation of a rat liver mitochondrial preparation, and (C) the product of a 30-min incubation of substrate only. Peaks: 1 = phenylacetaldehyde; 2 = benzaldehyde; R = reagent, PFPH and IS. Internal standard, aldrin.

solution in ethanol. A small amount of acetic acid was added to the reaction solution in order to accelerate the condensation reaction. Prior to injection into the gas chromatograph, any excess of PFPH was easily eliminated by washing with 1 ml of 6 *N* hydrochloric acid, to minimise damage to the ECD.

A series of standards, ranging in concentration from 2 to 20 nmoles of benzaldehyde and from 5 to 50 nmoles of phenylacetaldehyde per 4 ml of *n*-hexane containing IS was converted to the corresponding pentafluorophenylhydrazone and was separated by GC. Standard calibration curves were prepared from the data obtained by plotting peak height ratios of hydrazone—aldrin against the concentration of each aldehyde, and showed a linearity passing through the origin. The concentration of aldehyde generated in an enzyme reaction on a mitochondrial preparation was determined by using these curves.

To determine the optimal substrate concentration for the incubation mixture, assays were performed using different concentrations of benzylamine and β-phenylethylamine. Linear Lineweaver—Burk plots were obtained with observed K_m values of 0.38 m*M* for benzylamine and 0.20 m*M* for β-phenylethylamine. In the mixed substrate experiments, the concentration of substrate was fixed at 4 m*M* for benzylamine and at 2 m*M* for β-phenylethylamine, and MAO activities of mitochondrial preparations from rat liver were measured

according to the procedure described in Experimental. Fig. 1 shows a set of three chromatograms obtained using the standard assay procedure.

To investigate the effect of different amounts of enzyme on the rate of aldehyde production, we performed a series of incubations containing various quantities of the mitochondrial preparation and linear calibration curves were obtained up to 1.1 mg protein/0.1 ml of the enzyme concentration for benzylamine and β-phenylethylamine.

The precision of our method was determined by five repeated assays on an identical mitochondrial preparation according to the standard procedure. A standard deviation of 1.6% for benzylamine, 1.5% for β-phenylethylamine and mean aldehyde production rates (nmoles/mg protein/min) of 0.16 for benzylamine and 0.97 for β-phenylethylamine were obtained, respectively.

Using a mixture of two substrates, we examined the inhibitory effect on MAO activity. The reaction rates for both substrates were simultaneously examined by the standard procedure at five different benzylamine concentrations and constant β-phenylethylamine concentration (Fig. 2A). The ratios of the amounts of both aldehydes formed are illustrated in Fig. 2B. Plotting of the ratios against five different benzylamine concentrations shows a linear curve passing through the origin.

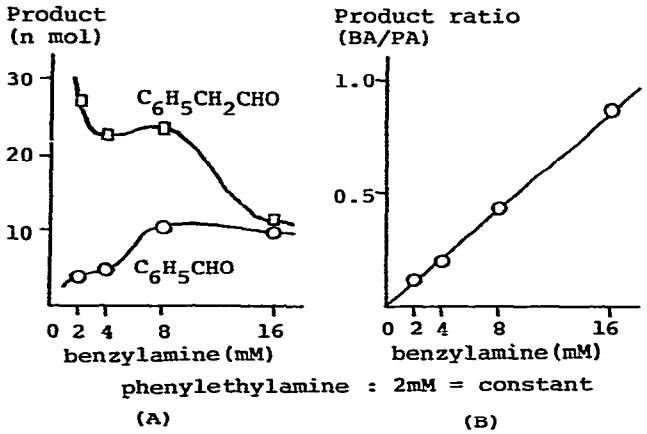

Fig. 2. Effect of substrate concentration ratio on the enzymatic reaction.

It seems likely that this technique could be applied to the analysis of MAO activity in serum and platelet samples. The method with mixed substrates is expected to offer an interesting means for studies on substrate specificity, differentiation of iso-enzymes and the simultaneous determination of some co-existing enzymes.

REFERENCES

1 J.P. Johnston, Biochem. Pharmacol., 17 (1968) 1285.
2 M.D. Houslay and K.F. Tipton, Biochem. Pharmacol., 24 (1975) 627.
3 M.D. Houslay, N.J. Garrett and K.F. Tipton, Biochem., Pharmacol., 23 (1974) 1937.
4 T. Egashira, Y. Kuroiwa and K. Kamijo, Arch. Biochem. Biophys., 191 (1978) 714.

5 K.F. Tipton and I.P.C. Spires, Biochem. J., 125 (1971) 521.
6 T. Nagatsu and K. Yagi, J. Biochem., 60 (1966) 219.
7 R. Lewinsohn, Clin. Chim. Acta, 81 (1977) 247.
8 H. Kochli and J.P. von Wartburg, Anal. Biochem., 84 (1978) 127.
9 M. Harada, K. Mizutani and T. Nagatsu, J. Neurochem., 18 (1971) 559.
10 H. Yamada, H. Suzuki and Y. Ogura, Advan. Biochem. Psychopharmacol., 5 (1972) 185.
11 M. Harada and T. Nagatsu, Anal. Biochem., 56 (1973) 283.
12 G. Nakano, M. Harada and T. Nagatsu, Biochim. Biophys. Acta, 341 (1974) 366.
13 K. Shimozato, T. Kawai, M. Hino, H. Kuzuya and T. Nagatsu, Arch. Oral Biol., 21 (1976) 495.
14 S. Takahashi and T. Karasawa, Clin. Chim. Acta, 62 (1975) 393.
15 R.E. McCaman, M.W. McCaman, J.M. Hunt and M.S. Smith, J. Neurochem., 12 (1965) 15.
16 B.A. Callingham and R. Laverty, J. Pharmacol., 25 (1973) 940.
17 B.J. Browne, C.J. Fowlor, and B.A. Callingham, J. Pharm. Pharmacol., 30 (1978) 573.
18 M. Jain, F. Sands and R.W. von Korff, Anal. Biochem., 52 (1973) 542.
19 P.H. Wu and L.E. Dyck, Anal. Biochem., 72 (1976) 637.
20 L. Demisch, H.J. Bochnik and N. Seiler, Clin. Chim. Acta, 70 (1976) 357.
21 F.F. Farris, E.O. Magarian and F.O. Slininger, Anal. Biochem., 88 (1978) 495.
22 G.H. Hogeboom, W.C. Schneider and G.E. Pallade, J. Biol. Chem., 172 (1948) 619.
23 O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem., 193 (1951) 265.
24 K. Kobayashi, M. Tanaka, S. Kawai and T. Ohno, J. Chromatogr., 176 (1979) 118.