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IDENTIFICATION BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY OF AN ADDUCT BETWEEN PURE PIG PLASMA BENZYLAMINE OXIDASE AND THE INHIBITOR 3,5-DIETHOXY-4-AMINOMETHYLPYRIDINE

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3,5-Diethoxy-4-aminomethylpyridine (B24) interacts with pure pig plasma benzylamine oxidase (BAO), giving a Schiff base with the carbonyl active site. This Schiff base was reduced, isolated by chemical hydrolysis of the enzyme, purified by HPLC and identified by gas chromatography-mass spectrometry (GC-MS) after derivatization. The isolated B24 adduct had the same absorption spectrum, retention time on HPLC and GC and the same mass spectrum as B24-pyridoxamine. B24, which is a reversible enzyme inhibitor, is also a weak substrate and competes with benzylamine, which is the best substrate, for the active site. These results further indicate the presence of pyridoxal-phosphate covalently linked to the pig plasma benzylamine oxidase and involved in the active site of this enzyme.

KEY WORDS: Amine oxidase inhibitors, benzylamine oxidase, semicarbazide sensitive amine oxidase, pyridoxamine derivative mass-spectrometry, pyridoxal-phosphate, picolylamine derivative

INTRODUCTION

Recently a new series of selective active-site directed inhibitors of benzylamine oxidase has been described.[1] One of these, 3,5-diethoxy-4-aminomethylpyridine (B24) is a selective reversible inhibitor of pig plasma benzylamine oxidase (BAO) and of tissue-bound semicarbazide sensitive amine oxidases with high affinity for benzylamine

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ABBREVIATIONS: BAO = benzylamine oxidase; PB24 = B24-pyridoxamine; PPB24 = B24-pyridoxamine-5-phosphate; GC-MS = gas chromatography mass spectrometry; MBTFA = N-methyl-bis-trifluoroacetamide; MSTFA = N-methyl-N-trimethylsilyl-trifluoroacetamide; DTDE = 2-hydroxy-ethyl-disulphide; B24 = 3,5-diethoxy-4-amino-methylpyridine; DAO = diamine oxidase; PLP = pyridoxal-phosphate; HPLC = high performance liquid chromatography; CI = chemical ionization; EI = electron impact; CF-FAB = continuous flow FAB; Bz.SSAO = semicarbazide-sensitive amine oxidase with high affinity for benzylamine

(Bz.SSAO).[2-4] B24 appears to be selective for Bz.SSAO enzymes, with a very low affinity for other enzymes such as diamine oxidase (DAO), mitochondrial monoamine oxidase (MAO A and B), lysyl oxidase.[1]

This paper describes the spectrophotometric interaction of B24 with pure pig plasma benzylamine oxidase and the identification of the reaction product.

EXPERIMENTAL PROCEDURE

Materials

Pyridoxal-5-phosphate (PLP), methanol and ethyl acetate Lichrosolv, sodium borohydride and phenyl hydrazine hydrochloride were purchased from Merck (Darmstadt, Germany), pyridoxal hydrochloride, *N*-methyl-bis-trifluoroacetamide (MBTFA) and *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) were purchased from Fluka (Buchs, Switzerland), Sep-Pak C18 and Sep-Pak silica cartridge were from Waters Association (Milford, Ma., USA) and benzylamine hydrochloride and pig kidney diamine oxidase were from Sigma (St. Louis, Mo., USA). The product 2-hydroxy-ethyl-disulphide (DTDE) was purchased from Aldrich (Milan, Italy). 14C-Benzylamine hydrochloride (54 mCi/mmole) was purchased from Amersham (Buckingamshire, U.K.).

The HPLC column Nucleosil 10C18 ET 300/8/4 was purchased from Macherey-Nagel (Dueren, Germany), the GC column DB 5 (25 m x 0.25 mm, 0.25 μm) was from J&W. (Folsom, Ca., USA).

Water was prepared with a Milli-Q-water purification system Milli-RO15 (Millipore, El Paso, Tx., USA).

Pure B24 in the form of the dihydrochloride (B24.2HCl) was prepared according to Bertini *et al.*[1]

All other reagents were of pure analytical grade.

Preparation of the enzyme

Pig plasma benzylamine oxidase was purified and crystallized according to Buffoni and Blaschko.[5]

Protein determination

The protein content of the samples was measured by determining the ultraviolet absorption at 215 and 225 nm respectively as described by Waddell and Hill.[6]

Preparation of the standards

B24-pyridoxamine (PB24). B24-pyridoximine was prepared by reacting 0.2 mmoles of pyridoxal hydrochloride with 0.2 mmoles of B24.2 HCl in 4 ml of methanol to which 0.1 ml of 13.38 M ammonia was added. The solution was vortexed and kept at

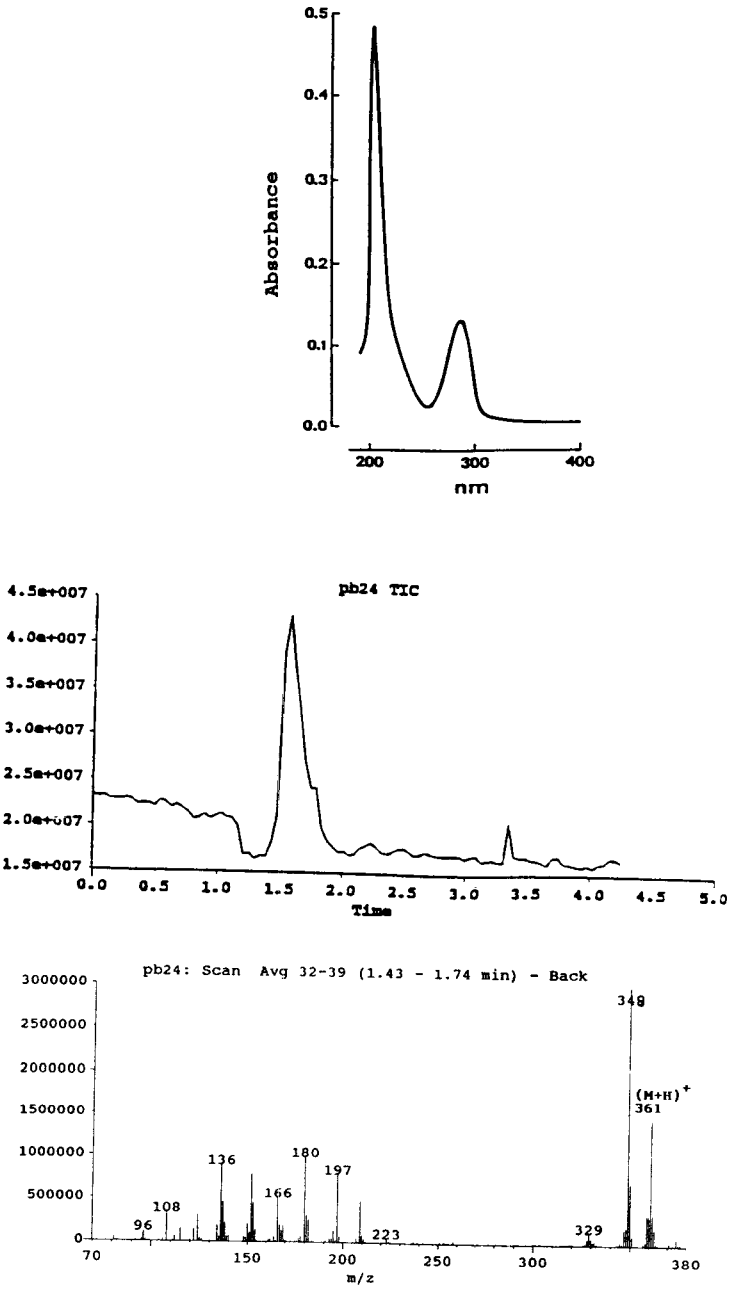

FIGURE 1 Absorption spectrum and CF-FAB spectrum of the standard B24-pyridoxamine (PB24). CF-FAB was obtained as described in Methods. The absorption spectrum was determined in methanol (PB24 0.8 μg/ml).

room temperature for 30 min. The precipitated B24-pyridoximine was recovered by centrifugation, washed twice with 5 ml of cold methanol and then dried under vacuum.

B24-pyridoxamine (PB24) was obtained by reduction with sodium borohydride: 0.003 mmoles of B24-pyridoximine in 1 ml of methanol and 1 ml of water was reduced with 10 mg of sodium borohydride. The mixture was shaken in a vortex, evaporated at reduced pressure and then 2 ml of water and 0.3 ml of 1 N hydrochloric acid were added to remove the excess of borohydride. The mixture was then brought to pH 10 with 25% (w/w) ammonia and the product extracted with 5 ml of ethyl acetate.

The organic phase was dried under vacuum and the PB24 was analyzed in the spectrophotometer, by HPLC and by CF-FAB. PB24 has an absorption maximum at 286.5 nm in methanol. The absorption and CF-FAB spectra are shown in Figure 1.

B24-pyridoxamine-5-phosphate (PPB24). 0.2 mmoles of PLP were reacted with 0.2 mmoles of B24.2 HCl in 4 ml of methanol to which 0.1 ml of 13.38 M ammonia was added. The solution was shaken in a vortex and left for 30 min at room temperature. The precipitated product was recovered by centrifugation, washed twice with 5 ml of cold methanol and dried under vacuum.

PPB24 was obtained by reduction with sodium borohydride: 0.0025 mmoles of B24-pyridoximine-phosphate in 1 ml of methanol and 1 ml of water was reduced with 10 mg of sodium borohydride. The mixture was vortexed and evaporated at reduced pressure, 2 ml of water was added and it was passed through 2 columns of Sep-Pak silica (one above the other) which were previously equilibrated with 4 ml of methanol and 4 ml of water. PPB24 was eluted with methanol and analyzed in the spectrophotometer and by HPLC. PPB24 has an absorption maximum in methanol at 286.5 nm.

Silylation procedure

In 1 ml vials the dried material eluted from HPLC at the same retention time as PB24 was derivatized with 40 μl of a mixture of 4:1 MSTFA to MBTFA (v/v) for 60 min at 70°C. Different amounts of dried standard PB24 eluted by HPLC (μg, 500, 100, 50 ng) were derivatized in 40 μl of the same mixture.

Equipment and procedures

A Perkin-Elmer Lambda 5 spectrophotometer and a LKB 2150 HPLC were used.

FAB spectra were obtained with a VG 7070 EQ mass spectrometer using Xenon at 8 keV. The CF-FAB was used with acetonitrile:water:DTDE (75:26:1) containing a 1% saturated aqueous solution of oxalic acid as a matrix at a flow rate of 3.5 μl/min.

EI and positive CI spectra were obtained in a Varian GC-MS system formed by a Varian 3400 gas chromatograph coupled to a Saturn III ion trap detector. Positive CI was obtained with isobutane as reagent gas. The oven temperature was 110°C for 2 min and then increased up to 300°C at the rate of 15°C/min. The injector and transfer line temperatures were 230°C. The gas chromatographic column was DB 5 (30 m × 0.25 mm, 0.25 μm).

HPLC analysis was carried out with a Nucleosil 10C18 ET 300/8/4 column and 90% methanol (v/v) in water as mobile phase. Peaks were detected at 280 nm.

Under these conditions PB24 had a retention time of 5 min 6 s ± 6 s (mean ± s.e. of 17 determinations) and a linear correlation was obtained (r = 0.999) between the peak heights and the amount of PB24 (ng 25–50–100–200).

RESULTS

(a) Spectrophotometric interaction of B24 with BAO

When B24 was added to a solution of BAO in M/15 sodium phosphate buffer pH 7.4 at 37°C there was a sudden variation in the absorption spectrum of the enzyme with the formation of a peak at 530 nm.

The maximum absorption value of this peak was obtained when B24 was in constant molar ratio with the enzyme. This peak gradually disappeared if the solution was kept at 37°C for a long period even in the presence of a large excess of B24, and a shoulder was formed around 360 nm which gradually changed into a peak at 340 nm. The maximum absorption at 340 nm was reached when B24 had reacted in a constant molar ratio with the enzyme and was stable for a long period in the presence of an excess of B24.

A linear relationship was obtained at 530 nm and at 340 nm between the absorbance and the enzyme concentration (r 0.993, statistically significant).

(b) Determination of the amine/enzyme molar ratio

The characteristic spectrophotometric variation produced by B24 permitted the titration of the reactive site of the enzyme.

In 1 ml cuvettes (1 cm light path) containing a known molar enzyme concentration a small volume of a solution containing B24 in amounts corresponding to a fraction of the molar enzyme concentration was added every 5–10 min and the spectral variations were recorded (Figure 2). The final molar ratio of B24 to enzyme was 0.97.

Very close results were obtained by titration with phenylhydrazine according to Buffoni and Ignesti[7] and with 14C-benzylamine.

When 14C-benzylamine was used the enzyme was reacted with 14C-benzylamine under anaerobic conditions (Argon). In an anaerobic tube with a lateral well, 7 mg of enzyme in 2.7 ml of M/15 sodium phosphate buffer pH 7.4 were gassed for 5 min with Argon, then 0.3 ml of 10 mM 14C-benzylamine was added and reacted for 5 min at 37°C.

The solution was then reduced by the addition of sodium borohydride (10 mg). The reduced enzyme was precipitated with ammonium sulfate at 55% saturation, centrifuged and the precipitate was dialyzed against water. The radioactivity and the

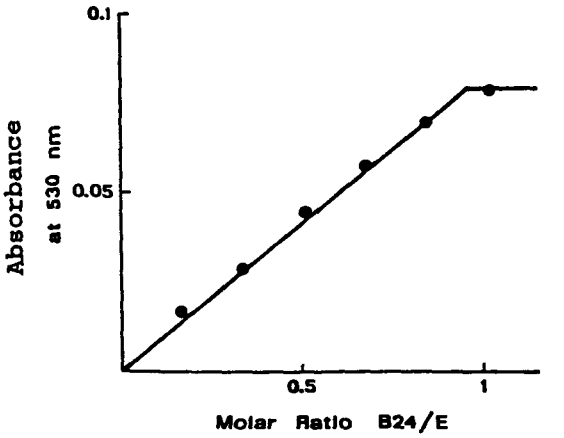

FIGURE 2 Titration of pig plasma benzylamine oxidase with B24. 10 μl of a 0.1 mM solution of B24 were added at 37°C to a 1 ml cuvette (1 cm light path) containing 1 ml of 5.9 μM BAO in M/15 sodium phosphate buffer pH 7.4 every 5 min. Before each addition the optical density at 530 nm was recorded using the M/15 buffer as reference blank. The B24/BAO molar ratio of 0.97 gave the maximum absorption.

protein content of the dialysate were measured: 0.92 moles of 14C-benzylamine were linked per mole of enzyme.

(c) Competition between B24 and benzylamine

Benzylamine itself increased the absorption spectrum of the enzyme at 530 nm and at 340 nm and diminished the increase in the optical density produced by B24 (Table 1).

B24 is a reversible inhibitor of the enzyme. Its inhibition is fully removed by dialysis[4] and it is also a weak substrate of the enzyme. The hydrogen peroxide formed in the reaction was followed using *o*-dianisidine under the conditions described by Buffoni and Raimondi.[8] The rate of oxidation of 1 mM B24 at 37°C and in air was 0.045 moles per mole of enzyme per min, a rate which was only 2% of the rate of oxidation of 1 mM benzylamine.

B24 is a primary amine very slowly oxidized by the enzyme, therefore the peak at 340 nm might be considered to be dependent on the formation of a Schiff base between the amine group of B24 and the co-enzyme. For this reason the B24-BAO reaction product was blocked by reduction and analyzed.

(d) Analysis of the B24-pig plasma benzylamine oxidase adduct

Experiments were carried out with pure pig plasma benzylamine oxidase and with PPB24 added to 12.5 mg of pig kidney DAO (internal standard).

For the analysis it was necessary to release the adduct from the protein. This was carried out by chemical hydrolysis under the previously described conditions[9] which were very good for releasing the phenylhydrazone of pyridoxal from the phenylhydrazone of pyridoxal-phosphate.

TABLE 1
Variation of the absorption spectrum of pig plasma benzylamine oxidase produced by the addition of benzylamine and of B24

B24/BAO molar ratio	Absorbance nm 530 without B*	nm 530 with B*	nm 340 without B*	nm 340 with B*
	a	b	a	b
0	0.084	0.124	0.160	0.184
0.05	0.085	0.116	–	–
0.10	0.090	0.114	0.160	0.175
0.20	0.108	0.125	0.160	0.167
0.30	0.126	0.137	0.160	0.174
0.50	0.142	0.157	0.175	0.180
0.70	0.159	0.157	0.185	0.197
0.90	0.169	0.162	0.200	0.202
1.10	0.168	0.162	0.205	0.202
Time				c
60 min				0.173
120 min				0.158
160 min				0.145
21 h 35 min				0.112

B* = benzylamine
(a) To a cuvette with 1 cm light path containing 1 ml 14.5 μM BAO in 50 mM sodium-potassium phosphate buffer pH 7.0 were added 5 μl of a 15 mM solution of B24 every 6 min and the spectra recorded every 3 min after the addition.
(b) To a cuvette with 1 cm light path containing 1 ml of 14.5 μM BAO in 50 mM sodium-potassium phosphate buffer pH 7.0 were added 500 nmoles of benzylamine and successively 5 μl of 15 mM solution of B24 every 6 min and the spectra recorded 3 min after the addition.
(c) The optical density at 340 nm gradually decreased in the presence of benzylamine whereas it was stable in the absence of benzylamine.

The yield for this procedure was obtained by hydrolyzing PPB24 in 4 ml of 0.027 M sulphuric acid in the presence of 1 mg of magnesium chloride and 1 mg of potassium chloride for 6 h at 115°C in closed hydrolysis tubes gassed with nitrogen. Under these conditions the yield of PB24 was 39% ± 4.3 (mean ± s.e. of 9 determinations) starting from 9, 18 and 26 μg of PPB24.

Pig plasma benzylamine oxidase was reacted with B24 either for 45 min or for 23 h at 37°C before reduction. Both procedures gave identical results.

A first sample of BAO (13.7 mg) in 0.9 ml of 0.05 M sodium-potassium phosphate buffer pH 7.0 was kept at 37°C for 45 min in the presence of 500 nmoles of B24. At the end of the incubation period 15 mg of sodium borohydride was added, the solution was kept at room temperature for 30 min and then dialysed against water at 4°C.

A second sample of BAO (20.3 mg) in 7 ml of 0.05 M sodium-potassium phosphate buffer pH 7.0 was reacted at 37°C with 10 μmoles of PB24 for 23 h, then reduced with sodium borohydride as described. The reduced enzyme was precipitated with ammonium sulphate at 55% saturation and the precipitate dialyzed against water at 4°C.

After 48 h of dialysis with two changes of water (1l), the solutions were placed in a hydrolysis tube and hydrolyzed in the presence of 0.027 M sulphuric acid, 1 mg of magnesium chloride and 1 mg of potassium chloride. Hydrolysis were carried out for 6 h at 115°C.

After hydrolysis the solutions were brought to pH 10 with ammonia and the released adduct extracted with 15 ml of ethyl acetate. The ethyl acetate fraction was evaporated under nitrogen and the dried material, solubilized in 200 μl of methanol, was analyzed by HPLC. It eluted at the same retention time as PB24. The eluted solutions were dried under nitrogen and vacuum and analyzed by GC-MS and in the spectrophotometer.

Figure 3 shows the variations in the spectra of BAO pruduced by B24, before and after reduction.

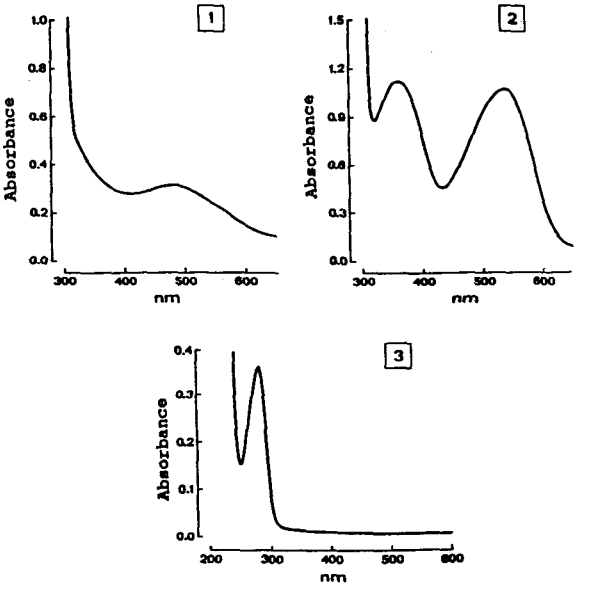

FIGURE 3 Absorption spectrum of pig plasma benzylamine oxidase after the addition of B24 and reduction. 50 μl of a 10 mM solution of B24 were added at 37°C to a 1 ml cuvette (1 cm light path) containing 0.9 ml of 76.3 μM BAO in 50 mM sodium-potassium phosphate buffer pH 7.0. (1) native enzyme; (2) 45 min after the addition of B24; (3) after reduction as described in the text and dialysis against water. The enzymic solution was diluted 35 times.

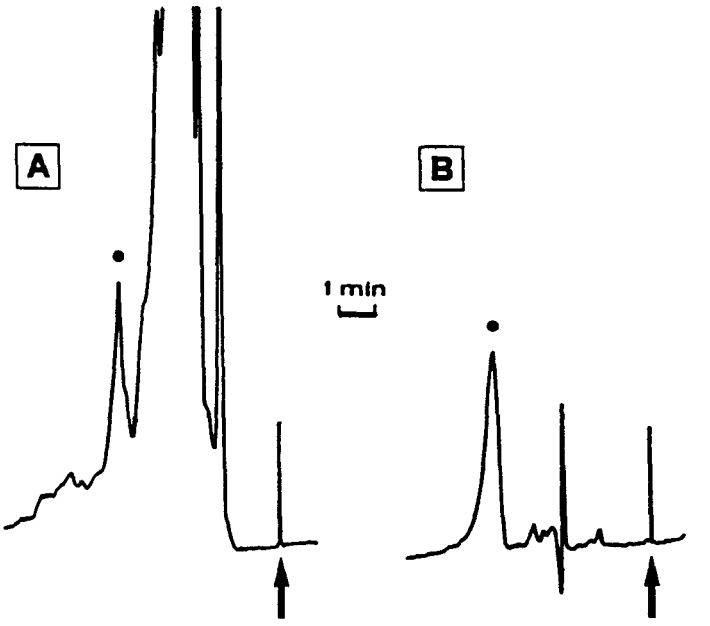

FIGURE 4 HPLC chromatogram of a B24-BAO adduct isolated by hydrolysis and Sep-Pak C18. For the conditions see Methods. At the arrow in (B), 5 μl of a methanol solution of PB24 containing 100 ng were injected, in (A) 20 μl were injected of the dried Sep-Pak C18 eluate of the second hydrolysis of 20.3 mg of pure pig plasma BAO reacted with B24 as described in the text and solubilized in 200 μl of methanol.

Two reagent blanks were carried out in parallel for the full procedure. Figure 4 shows a typical HPLC chromatogram.

The released adduct obtained from pig plasma benzylamine oxidase had the same retention time as PB24 on HPLC and the same spectrophotometric absorption maximum.

(e) Analysis of the B24-BAO adducts in GC-MS

The B24-BAO adducts and PB24 released by hydrolysis from the samples containing PPB24 and DAO eluted from HPLC were derivatized an analyzed in GC-MS as described in Methods.

The derivatization procedure used for silylation gave the following derivative of PB24:

Figure 5 shows the EI and CI spectra of the derivatized standard: the di-trimethylsilyl-monotrifluoroacetyl-derivative of B24-pyridoxamine (PB24), molecular weight 587. In EI the di-trimethylsilyl-monotrifluoroacetyl-derivative of B24-pyridoxamine gives two peaks at m/z 295 and 280 which indicates the loss of monotrifluoroacetyl-B24 (m.w. 292) and the formation of low molecular weight fragments with m/z 295 and 280 which are di-trimethylsilyl-derivative of pyridoxine.[10]

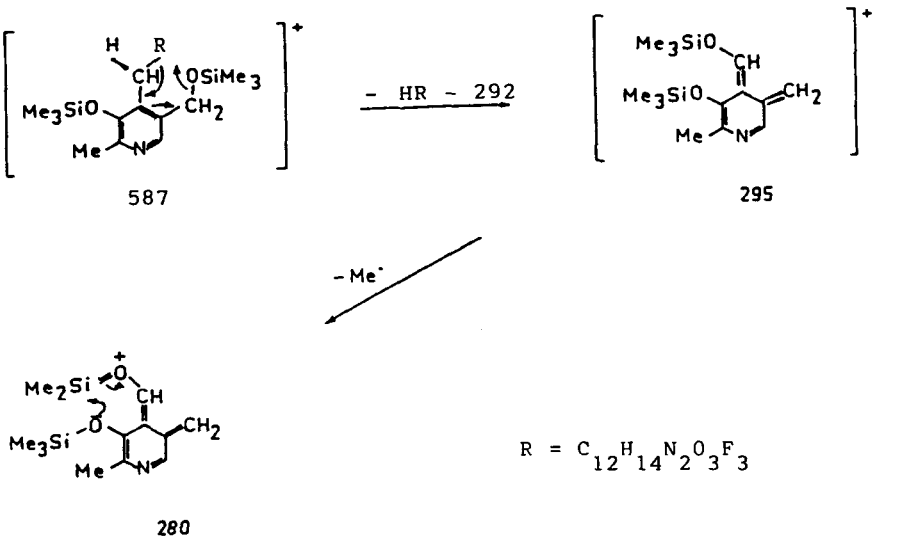

Figure 6 shows the CI spectra for the biological samples. In A and C are reported the total ion current and the ion chromatogram of m/z 588 and 498 of the adducts coming from hydrolysis of benzylamine oxidase (A) and from diamine oxidase to which internal standard PPB24 (C) has been added. B and D show the positive CI mass spectra of the trace peaks (A and C) at scan number 943. Trace A and B, which are an example of adduct analysis coming from benzylamine oxidase, are identical to traces C and D obtained with the internal standard.

DISCUSSION

B24 is a specific and reversible inhibitor of pig plasma benzylamine oxidase and of tissue-bound Bz.SSAO enzymes and also a weak substrate of pig plasma BAO.[1-4]

When benzylamine and B24 interact with pure BAO under aerobic conditions there is a sudden change in the absorption spectrum of the enzyme with a transient increase of the band at 530 nm and a more stable increase of the band at 340 nm.

The spectral variation at 530 nm disappears during the catalytic cycle of the reaction whereas the peak at 340 nm, which is slowly formed with B24, is stable. The variations of the spectrum produced by benzylamine are very modest in respect to those produced by B24 and this is a further indication that they correspond to transient forms of the enzyme-substrate complex, since benzylamine is a very fast substrate.

Both the spectral variations produced by B24 permit titration of the enzyme because they increase to a maximum value when a constant molar ratio for B24/BAO is reached. This molar ratio is very close to the active site content of the enzyme obtained by titration with phenylhydrazine[6] and 14C-benzylamine and with this enzymic preparation is unity.

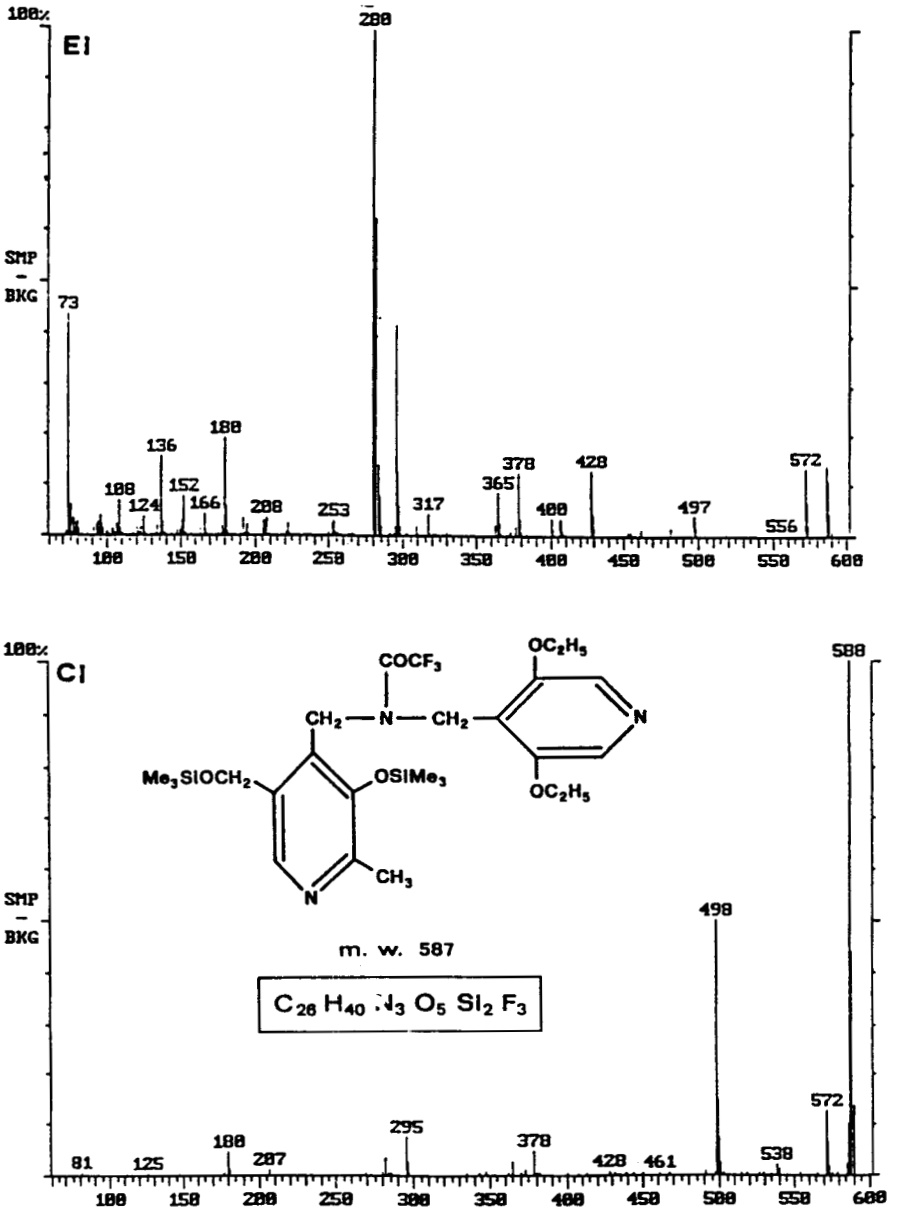

FIGURE 5 EI and positive CI spectra of di-trimethylsilyl-monotrifluoroacetyl derivative of B24-pyridoxamine.

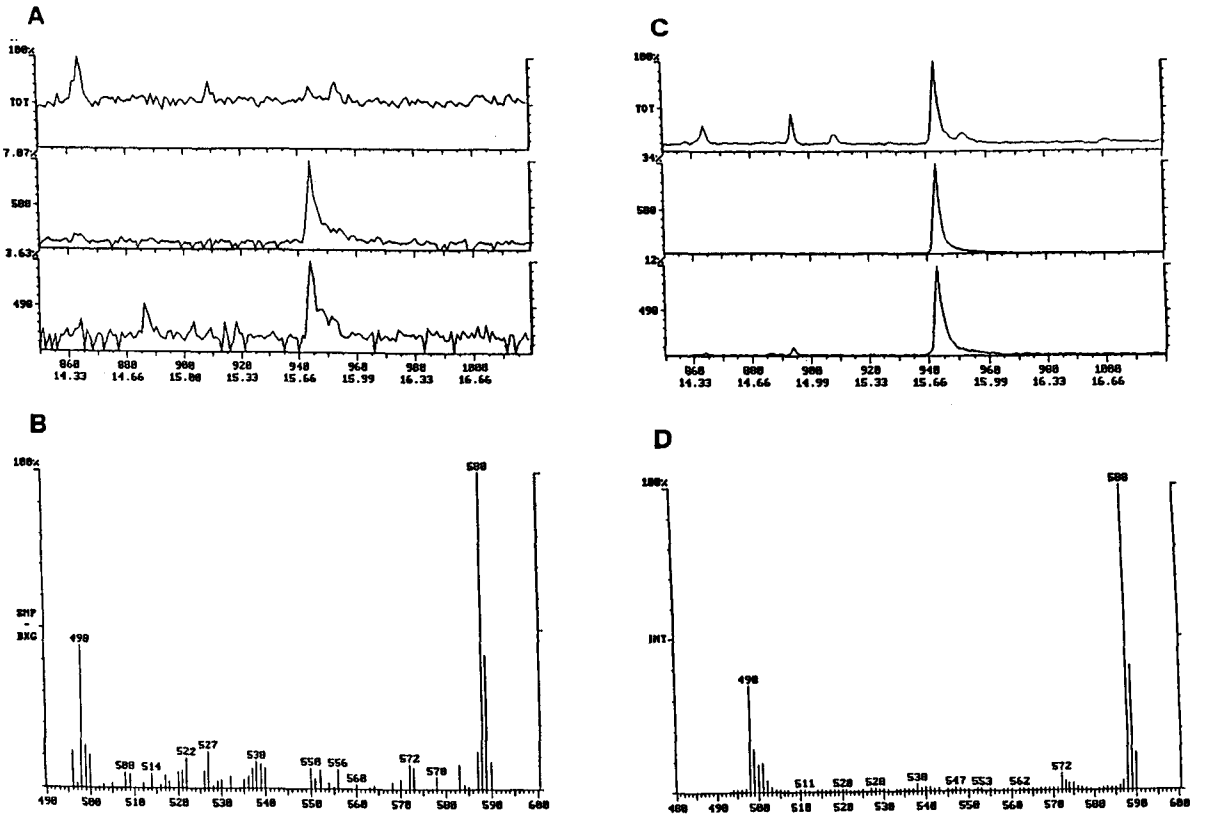

FIGURE 6 Total ion current, reconstructed ion chromatogram and mass spectra of the adducts isolated by pig plasma benzylamine oxidase and by PPB24. A and C: total ion current and reconstructed ion chromatograms at m/z 588 and 498 of the isolated adduct of the pure pig plasma benzylamine oxidase (A) reacted with B24 and hydrolyzed as described in the methods and (C) of the isolated adduct obtained by hydrolysis of PPB24 in the presence of protein (DAO) B and D: CI mass spectra recorded at the apex of the chromatographic peak, (B) adduct coming from pig plasma benzylamine oxidase, (D) from PPB24 and DAO.

The stable variation of the spectrum at 340 nm suggested the hypothesis that it might be an expression of the reaction of B24 with the carbonyl site of the enzyme. The data reported support this view because the adduct isolated by chemical hydrolysis after reduction, hypothesized PB24, actually gives the same retention time as PB24 on HPLC and gas chromatography and has the same mass spectrum. It seems therefore that B24 is reacting with the carbonyl site of BAO in the same way as benzylamine.

The main difference between B24 and benzylamine towards the enzyme is that B24 is only very slowly oxidized whereas the oxidation of benzylamine is fast.

In agreement with previous results in which the enzyme was reacted with phenylhydrazine,[11] these results further support the presence of pyridoxal in pig plasma benzylamine oxidase, a pyridoxal which is covalently linked to the enzyme in the form of pyridoxal-phosphate. It is released from the enzyme only under conditions able to split the phosphate group from PLP. The confirmed presence of PLP in pig plasma benzylamine oxidase is in contrast with the claimed presence of 6-hydroxy-dopa in this enzyme and in similar oxidases.[12–14]

On the other hand, the presence of 6-hydroxy-dopa is based on a method which is not suitable for showing the present of PLP in these enzymes, a demonstration which needs chemical hydrolysis.

Acknowledgements

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