Journal of Chromatography, 243 (1982) 123–129
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,839

DETERMINATION OF DIAMINE OXIDASE ACTIVITY BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

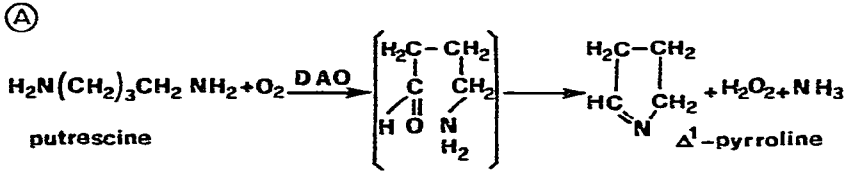
PIERGIORGIO PIETTA* and ALMA CALATRONI
Istituto di Chimica Organica ed Analitica, Facoltà di Agraria, Via Celoria 2, I-20133 Milan (Italy)
and
ROBERTO COLOMBO
Department of Biomedical Sciences and Technology, St. Raphael Hospital, Via Olgettina 60, I-20132 Milan (Italy)
(Received February 19th, 1982)

SUMMARY

A sensitive method for the assay of diamine oxidase is described, based on the separation by reversed-phase high-performance liquid chromatography of the products arising from the oxidation of the reagents (A) homovanillic acid, (B) 3,5-dichloro-2-hydroxybenzenesulphonic acid–4-aminoantipyrine and (C) pyridoxine–4-amino-N,N-diethylaniline. The analysis time is short and the assay is quantitative and reproducible. Because of its speed and high sensitivity and selectivity, the method is a useful alternative to the standard spectrophotometric assay.

INTRODUCTION

Diamine oxidase (diamine:oxygen oxidoreductase, deaminating, E.C. 1.4.3.6) (DAO) from pea seedlings oxidizes short-chain aliphatic diamines[1], such as putrescine, to give an aminoaldehyde, ammonia and hydrogen peroxide. The aminoaldehyde cyclizes to give Δ^1-pyrroline and the overall reaction is



Many methods have been reported for the assay of DAO based on manometric[2] and colorimetric[3–6] techniques. All of these methods are time consuming, of low sensitivity or not adequately reproducible and quantitative.

High-performance liquid chromatography (HPLC) has potential for enzyme assays, because it is rapid, selective and sensitive. In a previous paper[7] we described a reversed-phase HPLC method for the assay of oxalate oxidase by coupling the en-

0021-9673/82/0000–0000/$02.75

zymatically produced hydrogen peroxide with peroxidase and homovanillic acid. This approach has been extended to the determination of DAO activity using both the fluorogenic homovanillic acid (A) and the chromogenic 3,5-dichloro-2-hydroxybenzensulphonic acid–4-aminoantipyrine (B)[8] and pyridoxine–4-amino-N,N-diethylaniline (C)[9].

Reagent B has been widely employed in clinical chemistry after its application by Trinder[8] to the enzymic determination of glucose. As alternative we investigated also reagent C, which is oxidized by hydrogen peroxide/peroxidase to a strongly UV-absorbing quinoneimine.

The sequences of the reactions are shown in Fig. 1.

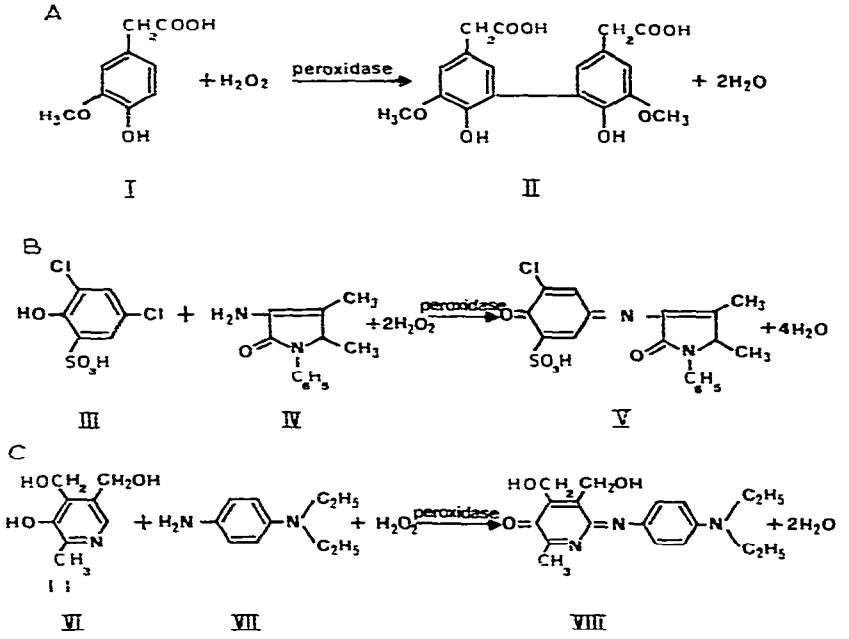


Fig. 1. Reagent A: I = homovanillic acid; II = 2,2′-dihydroxy-3,3′-dimethoxybiphenyl-5,5′-diacetic acid. Reagent B: III = 3,5-dichloro-2-hydroxybenzenesulphonic acid; IV = 4-aminoantipyrine; V = 4′-aminoantipyryl-3-chloro-5-sulphonic acid-1,4-benzoquinoneimine. Reagent C: VI = pyridoxine; VII = 4-amino-N,N-diethylaniline; VIII = 4′-amino-N,N-diethylanilyl-3,4-dihydroxymethyl-6-methyldihydropyridine-2,5-dioneimine.

The oxidized products are separated by reversed-phase HPLC and determined by fluorometric and ultraviolet detection.

EXPERIMENTAL

Apparatus

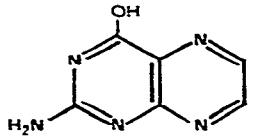
The chromatographic unit consisted of a Model 6000 solvent delivery system, a Model U6K Universal Injector, a Model 440 ultraviolet detector, a Model 730 Data Module (Waters Assoc., Milford, MA, U.S.A.) and a Jasco FP-110 spectrofluorimeter.

Ultraviolet absorption was measured at 254 nm; the fluorescence excitation and emission maxima were 315 and 425 nm, respectively.

A pre-packed stainless-steel column containing Bondapak C_{18} (30 cm × 4 mm I.D.; particle size 10 μm) was obtained from Waters Assoc. A pre-column of Bondapak C_{18} Corasil (2 cm × 4 mm I.D.) was used.

Materials

Reagent-grade diammonium hydrogen phosphate, 4-aminoantipyrine, pyridoxine hydrochloride, hydrogen peroxide, tris(hydroxymethyl)aminomethane were obtained from Merck (Darmstadt, G.F.R.), 3,5-dichloro-2-hydroxybenzenesulphonic acid (DHBS) from BDH (Poole, Great Britain), 4-amino-N,N-diethylaniline sulphate from Koch-Light (Colnbrook, Great Britain), homovanillic acid (HVA), nicotinic acid, 4-aminoantipyrine and peroxidase (Type II) from Sigma (St. Louis, MO, U.S.A.) and 2-amino-4-hydroxypteridine (AHP) from Fluka (Buchs, Switzerland).



2-Amino-4-hydroxypteridine

(AHP)

DAO was purified from 10-day germinated pea seedlings according to the method of Srivastava and Prakash[10]. Water was deionized and distilled and was filtered through Millipore membrane filters, pore size 0.45 μm (Millipore, Bedford, MA, U.S.A.). Methanol was of HPLC grade (Chromasolv; Riedel-De Haen, Hannover, G.F.R.).

Reagent A

Working reagent. HVA (0.17 mg/ml) and peroxidase (0.05 mg/ml) were dissolved in 0.1 *M* tris(hydroxymethyl)aminomethane buffer (pH 7.8). The reagent should be used within 12 h.

Reagent B

Buffer–peroxidase–4-aminoantipyrine reagent. 4-Aminoantipyrine (0.032 mg/ml) and peroxidase (0.6 mg/ml) were dissolved in 0.01 *M* diammonium hydrogen phosphate (pH 7.0).

DHBS reagent. 3,5-Dichloro-2-hydroxybenzenesulphonic acid (0.5 mg/ml) was dissolved in water.

Working reagent. The buffer–peroxidase–4-aminoantipyrine and DHBS reagents were mixed in the ratio 1:1. The resulting mixture should be used within 12 h.

Reagent C

Buffer–peroxidase–pyridoxine reagent. Pyridoxine hydrochloride (2 mg/ml) and peroxidase (1.72 mg/ml) were dissolved in 0.05 *M* diammonium hydrogen phosphate buffer (pH 7.0).

4-Amino-N,N-diethylaniline reagent. 4-Amino-N,N-diethylaniline sulphate (0.75 mg/ml) was dissolved in water.

Working reagent. The buffer–peroxidase–pyridoxine and 4-amino-N,N-diethylaniline reagents were mixed in the ratio 0.5:1. The mixture should be used within 12 h.

Nicotinic acid solution. Nicotinic acid (0.15 mg/ml) was dissolved in water.

AHP solution. 2-Amino-4-hydroxypteridine (0.082 mg/ml) was dissolved in 0.01 *M* sodium hydroxide solution (stable at 4°C for 4 weeks). A 1:100 dilution of this stock solution with 0.02 *M* sodium phosphate buffer (pH 7.25) was made before use.

Chromatographic conditions

Chromatography was performed at room temperature using 0.01 *M* diammonium hydrogen phosphate buffer–methanol. Before mixing, the buffer was adjusted to pH 4.9 with 20% phosphoric acid and filtered through a 0.45 μm membrane filter (Type HA, Millipore). The buffer to methanol ratios were 45:55, 40:60 and 25:75 for A, B and C, respectively. The flow-rates were 1.0 ml/min for A and B and 1.3 ml/min for C. Chromatographic peaks were monitored at 254 nm for B and C and fluorimetrically for A using an excitation filter of 315 nm and an emission cut-off filter of 425 nm.

Calibration graph

A 1-ml volume of working reagent A, B or C was subjected to reaction 0.0–0.20 μmol of hydrogen peroxide. After 15 min, 0.5 ml of AHP solution (A) or 0.15 ml of nicotinic acid solution (B and C) was added and replicate injections of 5 μl were made for each sample.

Diamine oxidase activity

A 5-μl volume of enzyme solution (1 mg/ml) and 1 μl of 0.1 *M* putrescine in 0.05 *M* phosphate buffer (pH 6.5) were added to 1 ml of working reagent A, B or C. The resulting mixture was incubated at 37°C for different times, then, 0.5 ml of AHP solution (A) or 0.15 ml of nicotinic acid solution (B and C) was added and 5 μl were injected into the chromatograph.

RESULTS AND DISCUSSION

Several mobile phases were investigated in order to develop a single system for all three reagents. Different ratios of 0.01 *M* diammonium hydrogen phosphate buffer (pH 4.9) to methanol gave the best results. The oxidized compounds and the components of each reagent were completely separated within 5 min.

Retention times and detection limits are shown in Table I.

Linear relationships between the peak-area ratios (II: AHP, V: nicotinic acid and VIII: nicotinic acid) and the amount of hydrogen peroxide were found over the range investigated (up to 0.20 μmol) and can be expressed by the following equations:

(A) $y = 10.534\,x + 0.094$; $r = 0.990$

(B) $y = 4.9512\,x + 0.115$; $r = 0.968$

(C) $y = 7.456\,x + 0.376$; $r = 0.978$

TABLE I

RETENTION TIMES AND DETECTION LIMITS

Reagent	*Compound*	*Retention time (min)*	*Detection limit (ng)*
A	I	—**	—**
	II	1.80	2.5
	AHP (I.S.)	2.60	0.1
B	III	4.52	250
	IV	4.27	3.3
	V	3.50	9.8
	Nicotinic acid (I.S.)	2.76	11
C	VI	2.39	18
	VII	2.79	36
	VIII	3.70	48
	Nicotinic acid (I.S.)	2.02	11

* For abbreviations, see Fig. 1 I.S. = internal standard.
** No fluorescence.

where y represents the amount of hydrogen peroxide (μmol) and x is the peak-area ratio.

The activity of diamine oxidase was calculated from the concentration of the enzymatically produced hydrogen peroxide, using the equation

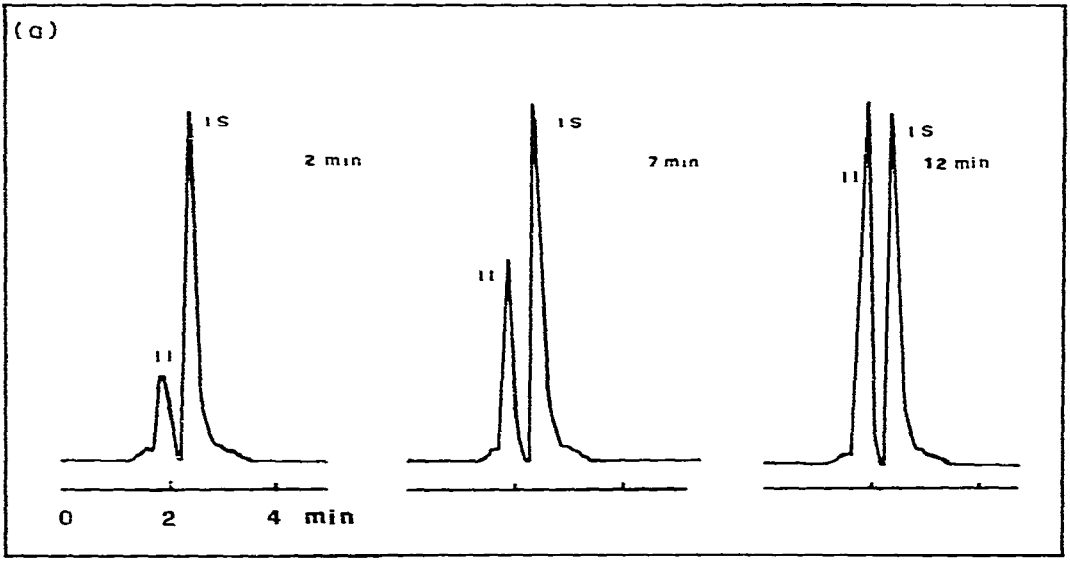
$$\text{activity (u/mg protein)} = \frac{\mu\text{mol } H_2O_2}{\text{time} \cdot \text{mg protein}}$$

In order to ensure favourable kinetics, diamine oxidase was incubated with a large excess of putrescine and the reaction was stopped by injecting the sample into the chromatograph. The results for incubations of 2, 7 and 12 min are shown in Fig. 2A, B and C, respectively.

The reproducibility of the method was determined by replicate analyses of the same samples and the activities were found to be 1.474 u/mg $\pm$ 1.2% for A, 1.7 u/mg $\pm$ 2.1% for B and 1.5 u/mg $\pm$ 2.4% for C.

CONCLUSIONS

The results illustrate the potential of HPLC for the assay of diamine oxidase. The sample preparation is minimal; the analysis time is short (5 min) and the oxidized products are completely separated from the reagents, thereby eliminating any interferences. Reagent A was found to be preferable in that it can be prepared rapidly and allows more accurate and sensitive detection.


(a)
IS
II
2 min
7 min
12 min
0
2
4 min

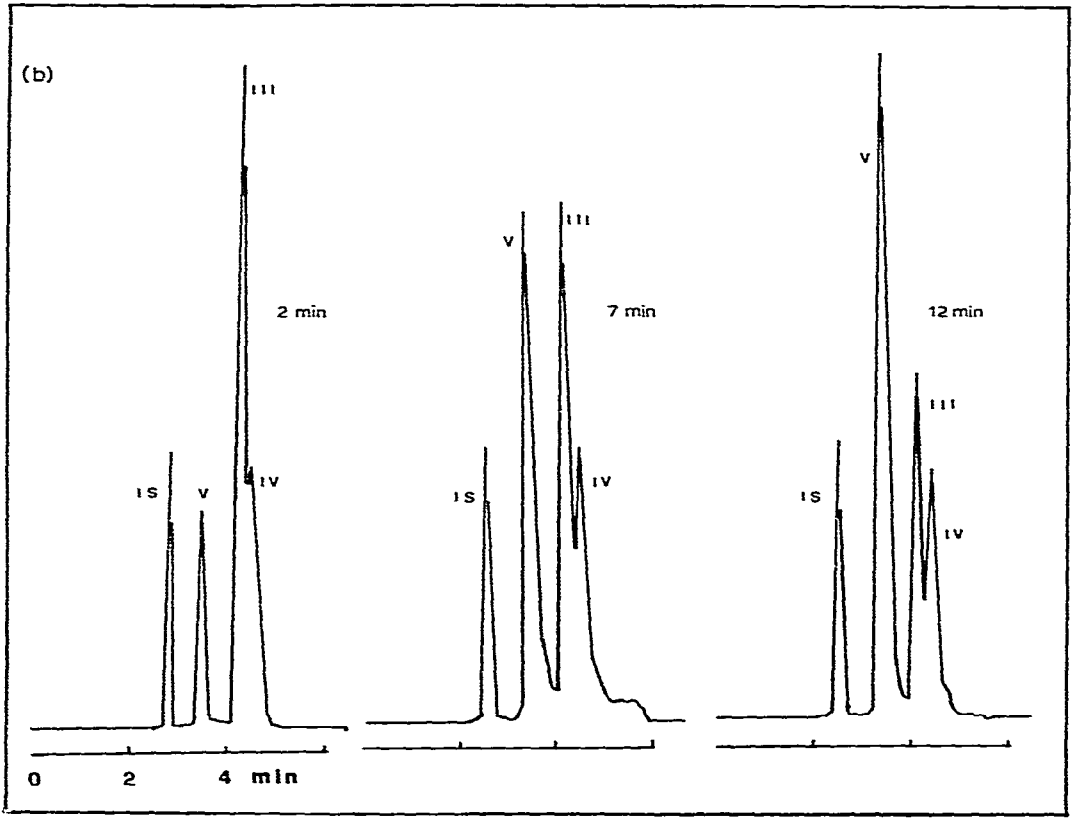

(b)
III
V
IS
IV
2 min
7 min
12 min
0
2
4 min

Fig. 2.

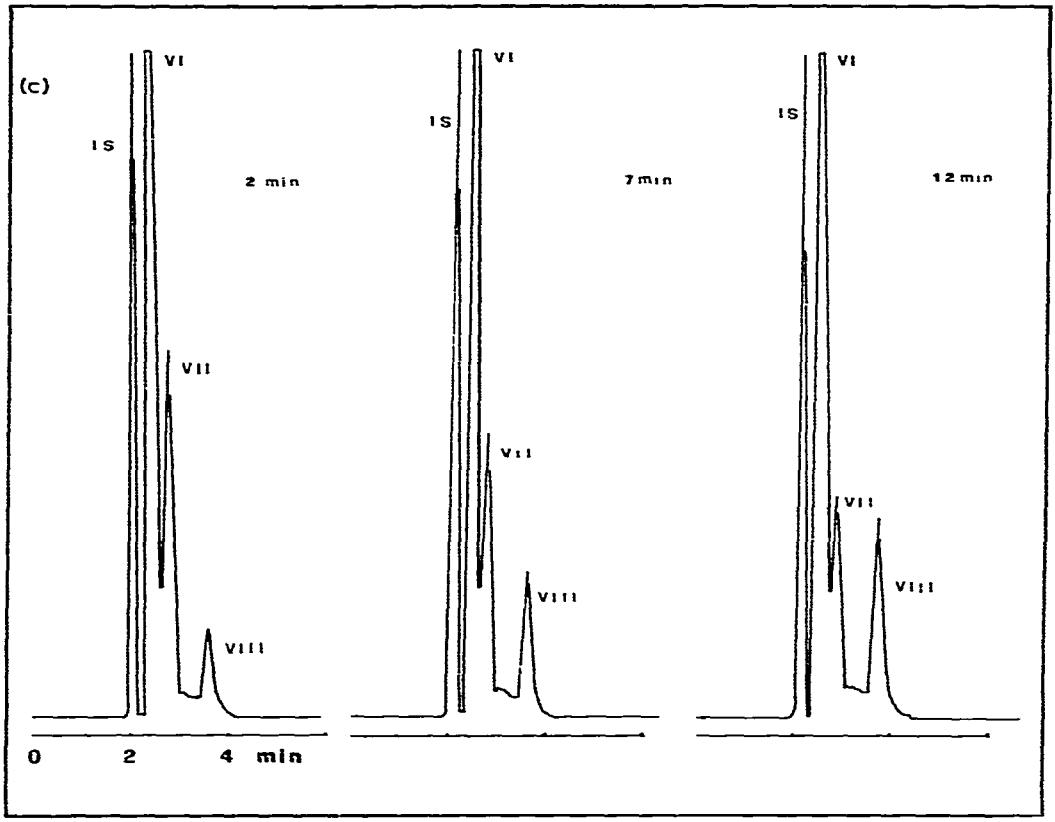


Fig. 2. Chromatograms of diamine oxidase incubated with putrescine for 2, 7 and 12 min. (a) II = 2,2'-Dihydroxy-3,3'-dimethoxybiphenyl-5,5'-acetic acid; I.S. = 2-amino-4-hydroxypteridine. Fluorescence detection (315/425 nm). (b) III = 3,5-Dichloro-2-hydroxybenzenesulphonic acid; IV = 4-aminoantipyrine; V = 4'-aminoantipyril-3-chloro-5-sulphonic acid-1,4-benzoquinoneimine; I.S. = nicotinic acid. Ultraviolet detection (254 nm). (c) VI = Pyridoxine; VII = 4-amino-N,N-diethylaniline; VIII = 4'-amino-N,N-diethylanilyl-3,4-dihydroxymethyl-6-methyldihydropyridine-2,5-dioneimine; I.S. = nicotinic acid. Ultraviolet detection (254 nm).

REFERENCES

1 J. M. Hill and P. J. G. Mann, *Biochem. J.*, 91 (1964) 171.
2 T. A. Smith, *Phytochemistry*, 11 (1972) 899.
3 B. Holmsted, L. Larsson and R. Tham, *Biochim. Biophys. Acta*, 48 (1961) 277.
4 W. G. Bardsley, M. J. C. Crabbe and J. S. Shindler, *Biochem. J.*, 127 (1972) 875.
5 T. A. Smith, *Phytochemistry*, 13 (1974) 1075.
6 B. I. Naik, R. G. Goswani and S. K. Srivastava, *Anal. Biochem.*, 111 (1981) 146.
7 P. G. Pietta, A. Calatroni and M. Pace, *J. Chromatogr.*, 241 (1982) 409.
8 D. Barham and P. P. Trinder, *Analyst (London)*, 97 (1972) 142.
9 R. Strohecker and H. M. Henning, *Vitamin Assay*, Verlag Chemie, Weinheim, 1965, p. 131.
10 S. K. Srivastava and V. Prakash, *Phytochemistry*, 16 (1977) 189.