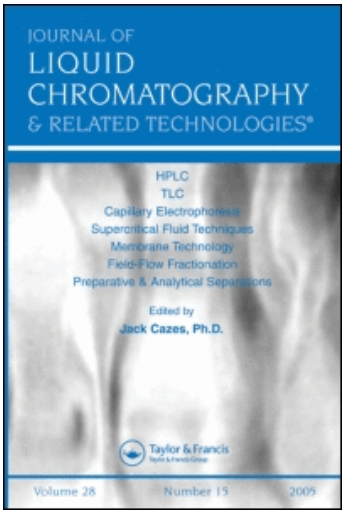

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Nissinen, Erkki and Linko-Löppönen, Solveig(1985) 'High-Performance Liquid Chromato- Graphic Assay for Kinetic and Inhibition Properites of Monoamine Oxidase form A', Journal of Liquid Chromatography & Related Technologies, 8: 2, 395 — 405
To link to this Article: DOI: 10.1080/01483918508067087
URL: http://dx.doi.org/10.1080/01483918508067087

JOURNAL OF LIQUID CHROMATOGRAPHY, 8(2), 395-405 (1985)

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR KINETIC AND INHIBITION PROPERITES OF MONOAMINE OXIDASE FORM A

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ABSTRACT

A rapid and convinient method to study the kinetic and inhibition properties of rat brain monoamine oxidase form A by high-performance liquid chromatography is described. The procedure consists of homogenization of the brain tissue, enzymatic reaction with or without inhibitors using 5-hydroxytryptamine as the substrate, termination of the reaction with perchloric acid, removal of protein by centrifugation and detection of reaction products at 280 nm after HPLC separation. The method was applied to determine K_m and V_{max} values of 5-HT for MAO-A and to study the inhibition properties of MAO-A using clorgyline and l-deprenyl as inhibitors.

INTRODUCTION

Monoamine oxidase (MAO; monoamine O_2:oxidoreductase EC 1.4.3.4) is the enzyme, which is responsible for the

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oxidative deamination of a variety of primary, secondary and tertiary amines. The enzyme has an important role in the regulation of the metabolism of catecholamines and 5-hydroxytryptamine (5-HT) in the brain and other tissues (1).

As defined by Johnston (2) MAO exists in two forms: MAO-A and MAO-B on the basis of their substrate selective inhibition by clorgyline and l-deprenyl. MAO-A preferetially deaminates 5-hydroxytryptamine and norepinephrine and is inhibited by low concentrations (nM) of clorgyline, while high concentrations (μM) l-deprenyl are needed to cause inhibition of the same magnitude. MAO-B deaminates benzylamine and β-phenylethylamine and is selectively inhibited at low l-deprenyl concentrations.

Several methods exist to assay MAO activity. These include spectrophotometry (3,4), fluorometry (5,6), polarographic (7) and radiometric techniques (8,9). The high sensitivity and specificity of the radiochemical methods has led to the wide use of these methods to study MAO activity although they include time consuming extractions and use of expensive isotopes.

Recently gas chromatography (GC) was introduced to assay MAO activity but these GC methods require derivatization procedures (10,11) before the products can be determined. High-performance liquid chromatography (HPLC) with fluoresence detection has been shown to be a novel method to assay MAO activity (12 - 13).

We have developed HPLC method for the assay of MAO-B activity by UV-detection (14) and we report here adaptation of this method to assay and to study the kinetic properties of MAO-A in rat brain.

MATERIALS AND METHODS

Reagents

5-hydroxytryptamine creatinine sulphate was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Clorgyline was obtained from May & Baker (Dagenham, UK) and l-deprenyl from Chinoin (Budapest, Hungary). Methanol, HPLC grade, was from Orion Pharmaceutical Co. (Espoo, Finland). All other chemicals were of analytical reagent grade.

Apparatus

The HPLC analyses were carried out with a combination of a Waters Model 6000 A pump, a Waters Intelligent Sample Processor (WISP) model 710 B (Waters Assoc., Millford , MA, USA) equipped with a 150 x 4.6 mm, 5 um Ultraspere-ODS column fitted with a 45 x 4.6 mm precolumn (Beckman Instruments, Fullerton, CA, USA). A Kratos Model 773 variable wavelength detector at 280 nm (Kratos Analytical Instruments, Ramsey, NJ, USA) was used for the detection of the eluted components. The mobile phase consisted of 0.1 M sodium phosphate and 20 mM citric acid in

20 % methanol, pH 3.2. The isocratic elution was performed at ambient temperature using flow rate of 1.5 ml min^{-1}.

Sample prepration

Han:Wist male rats weighing 100 - 150 g were killed with carbon dioxide, the brains were removed and homogenized 1:4 (w/v) in ice cold 10 mM potassium phosphate buffer, pH 7.4 containing 0.25 M sucrose. The homogenate was centrifuged for 15 min at 600 x g. The supernatant was centrifuged for 20 min at 6500 x g. The resulting pellet containing the crude mitochondrial fraction was washed twice in the homogenization medium and finally resuspended in the homogenization buffer and stored in small aliquots at -20°C until assayed. Protein determinations were carried out using a Bio-Rad protein assay kit (Bio-Rad Labs., Richmond, CA, USA).

Assay

The incubation mixture contained 0.14 M sodium phosphate, pH 7.4, 0.5 mM 5-HT and the mitochondrial preparation (about 0.5 mg protein) in a total volume of 0.25 ml. The mixture was incubated in open tubes for 30 min except for the study of time course at 37°C using a shaking waterbath. The reaction was terminated by the addition of 25 µl 4 M perchloric acid. Protein was removed by centri-

fugation and a 20 µl aliquot was injected into the liquid chromatograph.

The inhibition studies were performed preincubating the enzyme for 20 min with varying amounts (10 pmoles to 1 µmole) of clorgyline or l-deprenyl before the addition of the substrate 5-HT. The kinetic properties were studied using different amounts (20 - 200 µmoles) of the substrate 5-HT. V_{max} and K_m values were calculated using the Lineweaver-Burk double reciprocal plots.

RESULTS AND DISCUSSION

A typical chromatogram of MAO-A assay is shown in Fig. 1. The blank (Fig. 1A), incubation mixture without the enzyme, does not contain any peaks eluting after the substrate 5-HT (r_t = 2 min). Chromatogram resulting from the complete incubation mixture is presented in Fig. 1B. Peak I was identified as 5-hydroxyindoleacetaldehyde and peak II as 5-hydroxyindoleacetic acid (5-HIAA). The small amount of 5-HIAA was formed under described assay conditions by the action of aldehyde dehydrogenase present in the brain mitochondrial homogenate. The identity of the peak I as 5-hydroxyindoleacetaldehyde was futher confirmed using enzymatic peak shift technique by incubating the reaction mixture 10 min with 20 units aldehyde dehydrogenase and 1 mM NAD. This resulted in growing of the

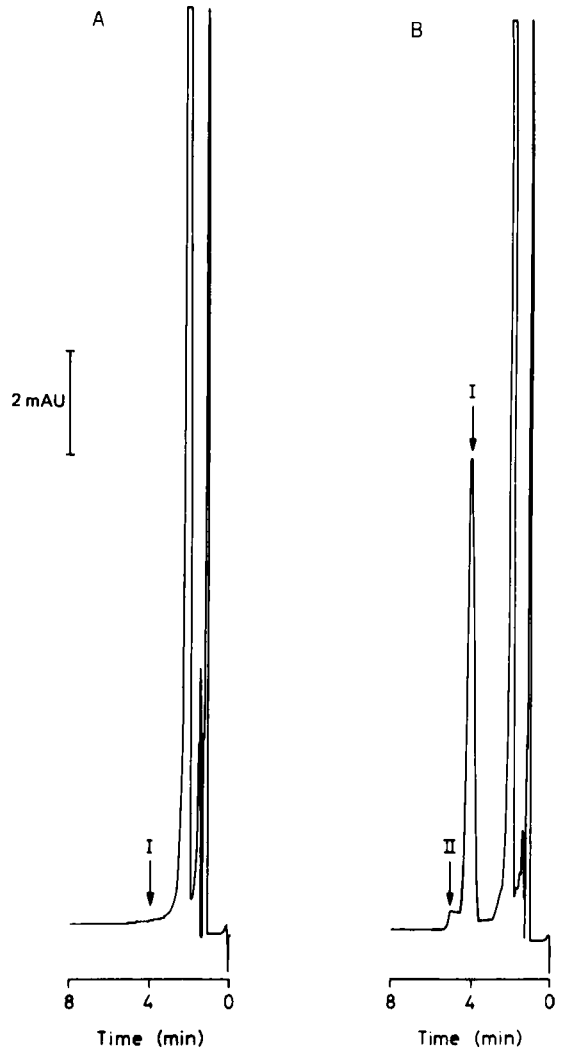

FIGURE 1. Chromatograms obtained with (A) blank incubation mixture and (B) the rat brain mitochondrial preparation. Peaks: I = 5-hydroxyindoleacetaladehyde, II = 5-hydroxyindoleacetic acid. Chromatographic conditions as described under materials and methods.

peak II (5-HIAA) with simultaneous disapperence of the peak I.

The rate of 5-hydroxyindoleacetaldehyde formation was linear for 30 min at 37°C as shown in Fig. 2. Linearity was also found up to 1 mg enzyme protein and aldehyde formation during 30 min incubation at 37°C. The inter-assay reproducibility (n = 6) was 4.5 % expressed as coefficient of variation.

The kinetic properties of MAO-A obtained using this procedure revealed the K_m value of 171 $\pm$ 29 μM for 5-HT and V_{max} value of 2.8 $\pm$ 0.3 nmoles product x mg protein^{-1} x min^{-1}. These values are in good agreement with those reported by others using rediochemical assays (16, 17).

Clorgyline and l-deprenyl are specific inhibitors for MAO-A and MAO-B respectively. The effect of these inhibitors on MAO-A activity in the presence of 0.5 mmolar 5-HT is presented in Fig. 4. The inhibition patterns show complete inhibition of MAO-A activity at the concentration of 100 nmoles of clorgyline, while 100 μmoles of l-deprenyl are needed to suppress MAO-A activity to the same level. These sigmoidal inhibition curves are identical to those obtained with radiochemical assays (9, 18) and with fluorimetric HPLC assays (13).

The described HPLC assay should prove to be very convenient means to study MAO activity in different tissues as well as to investigate the effects of various new drugs on MAO activity.

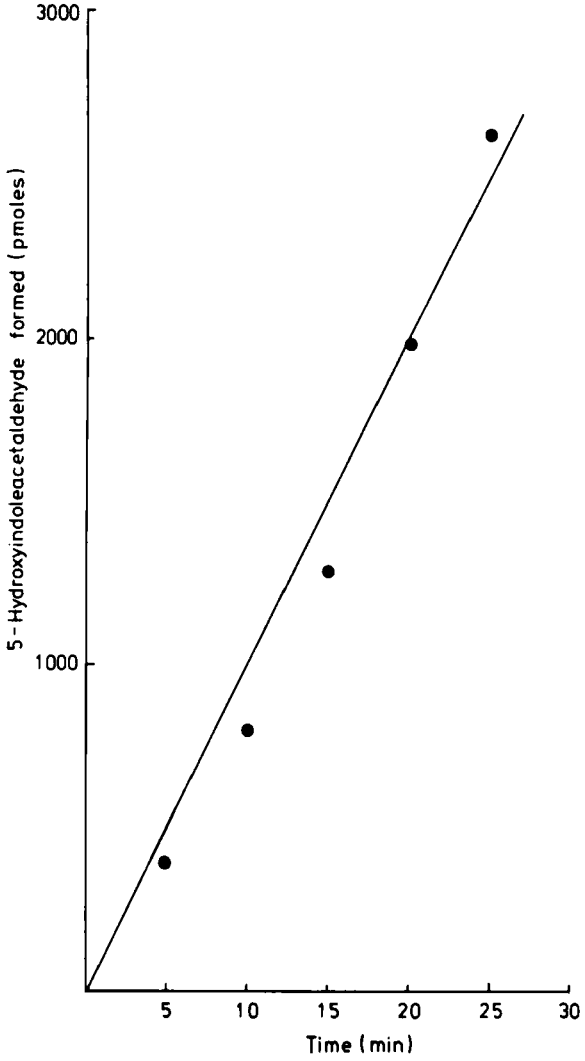

FIGURE 2. Time course of enzymatic deamination of 0.5 mM 5-HT in incubation mixture containing 0.5 mg enzyme protein.

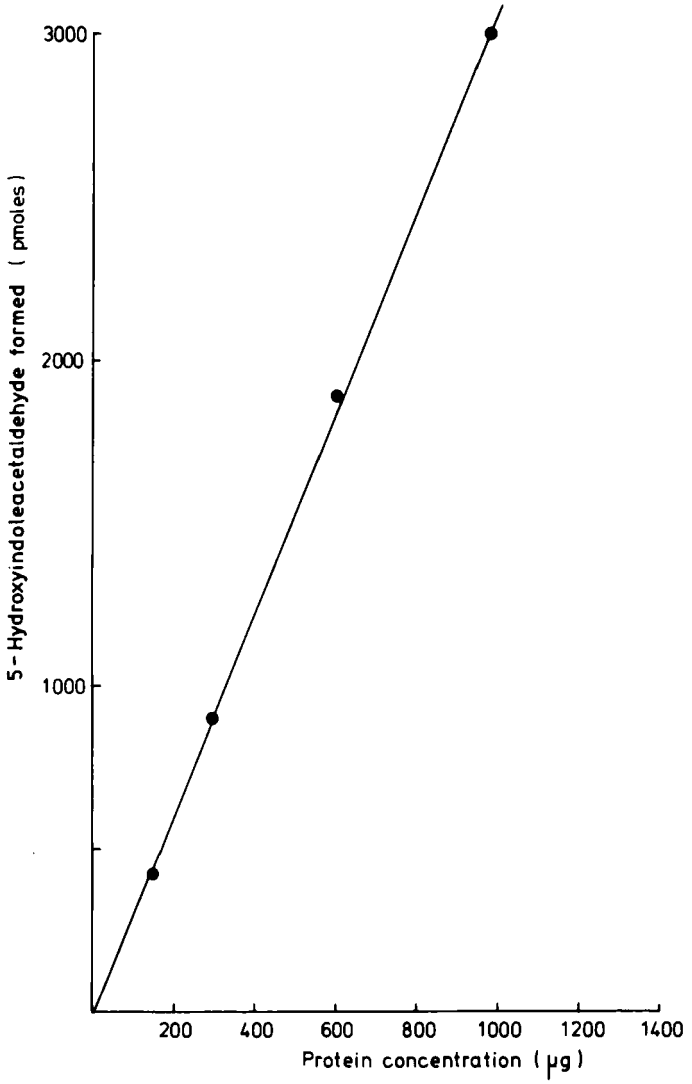

FIGURE 3. The linearity of MAO-A assay with rat brain mitochondrial fraction.

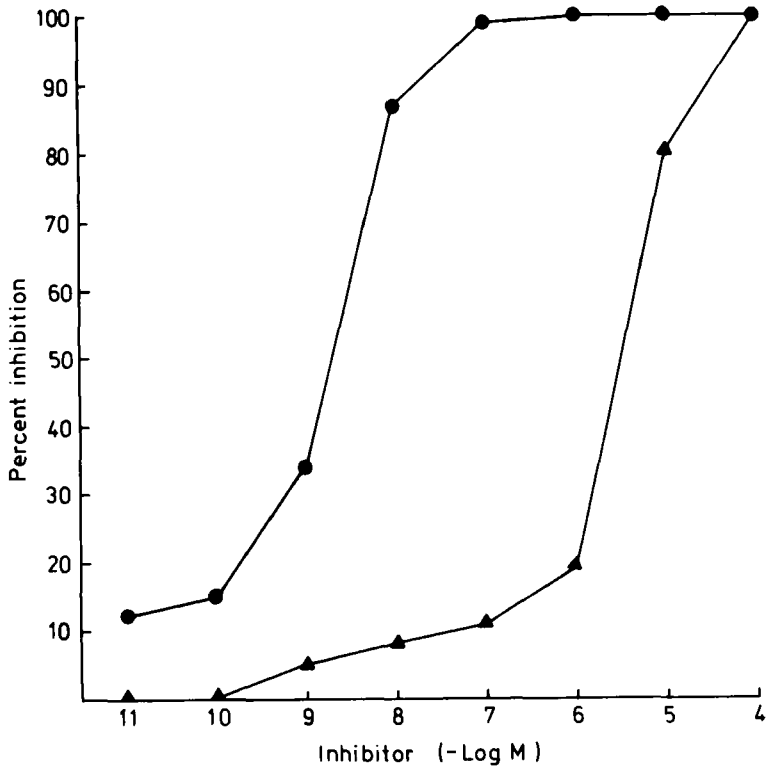

FIGURE 4. Inhibition of the deamination of 0.5 mM 5-HT by (●) clorgyline and (▲) l-deprenyl. Rat brain homogenates were preincubated for 20 min at 37°C with varying amounts of the appropiate inhibitor before assayed for activity. Values are expressed as mean (n = 3) of inhibition % with respect to samples preincubated without inhibitors.

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