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Note

Reversed-phase high-performance liquid chromatographic method for the measurement of polyamine oxidase activity

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The polyamines putrescine, spermidine and spermine are intracellular cations which are essential for the normal process of cellular proliferation and differentiation [1–3]. Ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (AdoMetDC) are the two principal enzymes involved in polyamine biosynthesis, and methods for their assay are well established [4–6]. ODC catalyzes the decarboxylation of ornithine to form putrescine which can be converted into spermidine and then spermine by successive additions of aminopropyl groups from decarboxylated S-adenosylmethionine, a product of AdoMetDC [7]. More recently, it has been recognized that spermine can be converted back into spermidine, and spermidine can be broken down to form putrescine by the "reverse pathway" of polyamine metabolism [8,9]. Each step in this degradation involves first an acetylation by the rate-limiting enzyme spermidine–spermine acetyltransferase (SAT) and second, oxidation by the enzyme polyamine oxidase (PAO) [8–10].

PAO was first purified and characterized from rat liver, and the original assay method for this enzyme utilized spermidine or spermine as substrate [11]. Because it was subsequently shown that the acetylated polyamines were preferred substrates for PAO [8,9], an alternative assay procedure was developed using N^1,N^{12}-diacetylspermine as substrate [12] Although this procedure offered a more sensitive method for assaying PAO, it required a lengthy dansylation of the reaction products and separation by thin-layer chromatography which made it cumbersome. Other procedures for the assay of PAO have been developed [11,13,14] but these too have certain disadvantages. Methods which detect the formation of hydrogen peroxide, a side product of the PAO reaction [11,13], are indirect and subject to error due to the many other oxidases present in the cell which can form this product and potentially interfere with the assay [14,15]. Recently a method for the assay of PAO has been published which uses an ion-

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exchange column to separate the substrate, N^1-acetylspermidine, from its reaction product putrescine and offers many advantages over previous methods [16]. Although this technique utilizes the preferred substrate for PAO and eliminates the need for radioisotopes, adequate separation of the reaction products by ion-exchange chromatography takes over 1 h and automation of this method requires the use of an amino acid analyzer A rapid colorimetric assay has been proposed using a bis(benzyl)polyamine analogue as substrate and is based on the measurement of benzaldehyde, which is formed by the action of PAO on this substrate [17]. A disadvantage of this method is that the bis(benzyl)polyamine analogues are not yet widely available. Furthermore, benzaldehyde is a potent activator of PAO [11] and the potential for product interference with the measurement of PAO activity has not been fully evaluated. Based on these considerations our laboratory has developed and standardized a more rapid assay for PAO utilizing a reversed-phase, ion-pairing high-performance liquid chromatographic (HPLC) method for the determination of polyamines which serves as the basis for this report.

EXPERIMENTAL

Chemicals

HPLC-grade acetonitrile and octanesulfonic acid (OSA) were obtained from Alltech Assoc. (Arlington Heights, IL, U.S.A.). The PAO inhibitor N^1,N^2-bis (2,3-butadienyl)-1,4-butane diamine (MDL 72527) was provided by Merrell Dow Pharmaceuticals (Cincinnati, OH, U.S.A.). All other chemicals were of reagent grade or better and were purchased from Sigma (St. Louis, MO, U.S.A.).

Animal treatment

Throughout these studies, male Sherman albino rats weighing 200–250 g were used. In addition to control rats used to standardize the PAO enzyme assay, an additional group of twenty rats were injected intraperitoneally with either saline or MDL 72527 in saline (20 mg/kg body weight) and then sacrificed one, two, four and seven days post-injection to assess the time course of PAO inhibition.

Polyamine oxidase assay

All enzyme assays were performed on cytosolic fractions of small intestinal or colonic mucosa taken from rats fasted overnight, by a modification of the original method described by Hölttä [11]. Although the highest specific activity of PAO has been localized to the light mitochondrial fraction in rat liver, over half of the total activity is found in the cytosol [11] and, therefore, this fraction was used for all subsequent experiments. Mucosa from colon and small intestine was scraped with a scalpel and homogenized in 5 volumes of 0.25 *M* sucrose–10 m*M* Tris, pH 7.4, and centrifuged at 100 000 *g* for 1 h to obtain the cytosolic fraction which was used for the enzyme source. The standard PAO reaction mixture

contained 0.1 *M* glycine, pH 9.5, 5 m*M* dithiothreitol, 250 μ*M* N^1-acetylspermidine and 250–400 μg of protein in a final volume of 250 μl. In addition, the reaction mixtures contained 0.56 m*M* aminoguanidine and 0 04 m*M* pargyline, known inhibitors of the enzymes diamine oxidase and monoamine oxidase, respectively, to inhibit degradation of the reaction product, putrescine, as recommended by Seiler *et al.* [12]. Previous studies have shown that concentrations of aminoguanidine and pargyline up to 1.0 and 0.1 m*M*, respectively, do not have inhibitory activity towards PAO [17]. The reaction was begun by addition of enzyme, incubated at 37°C for various lengths of time up to 60 min and stopped by the addition of 50 μl of 50% trichloroacetic acid. The reaction mixture was then transferred to a 1-ml syringe and filtered through a low-volume 0.45-μm Durapore membrane (Millipore, Bedford, MA, U.S.A.). Aliquots (25–100 μl) of filtrate were then injected onto a reversed-phase HPLC column for determination of N^1-acetylspermidine and putrescine levels.

Chromatographic system

The HPLC system consisted of two pumps, Models 6000A and M45, controlled by a Maxima 800 chromatography workstation and WISP 710A autosampler equipped with refrigeration unit from Waters Assoc. (Milford, MA, U.S.A.). Post-column derivatization of polyamines with *o*-phthalaldehyde (OPA) was accomplished with an Eldex piston pump (San Carlos, CA, U.S.A.) and derivatives were analyzed in a Model 2070 spectrofluorometer from Varian (Sugarland, TX, U.S.A.) with excitation and emission wavelengths of 340 and 455 nm, respectively. The detector output was stored and integrated on the Maxima system.

Components were separated on an Ultrasphere ion-pair column (5 μm particle size, 250 mm × 4.6 mm I.D.) from Beckman (Berkeley, CA, U S.A.) fitted with a Guard-Pak pre-column module and C_{18} cartridge from Waters according to the method of Seiler and Knödgen [18], using a gradient elution technique Solvent A consisted of a mixture of 0.1 *M* sodium acetate and 10 m*M* OSA adjusted to pH 4.5 with HPLC-grade acetic acid. Solvent B contained 0.2 *M* sodium acetate (pH 4.5)–acetonitrile (10:3, v/v) with 10 m*M* OSA. Optimal and rapid separation of compounds was obtained at a flow-rate of 1.0 ml/min using a linear gradient of 35% A and 65% B to 100% B over 10 min followed by 15 min of isocratic elution with solution B. The OPA reagent was made as previously published [18] and delivered at a flow-rate of 1.0 ml/min.

PAO kinetics, protein and time studies

The Michaelis constant, K_M, and maximal velocity, V_{max}, for PAO enzyme activity in the colon and small intestine were determined by varying the concentration of N^1 -acetylspermidine from 10 to 500 μ*M* in the reaction mixture and constructing a Lineweaver–Burk plot. In addition, the linearity of the reaction with respect to time and amount of enzyme used was determined by either stop-

ping the reaction at 5, 10, 15, 30 or 60 min or by varying the amount of cytosolic protein from 100 to 1000 μg per reaction.

RESULTS

Chromatographic determination of polyamines

A rapid and efficient separation of N^1-acetylspermidine from putrescine in the reaction mixture was obtained using a reversed-phase, ion-pairing HPLC technique [18,19]. Optimal results were obtained using a linear gradient of 35% A–65% B going to 100% B over 10 min followed by 15 min of isocratic elution.

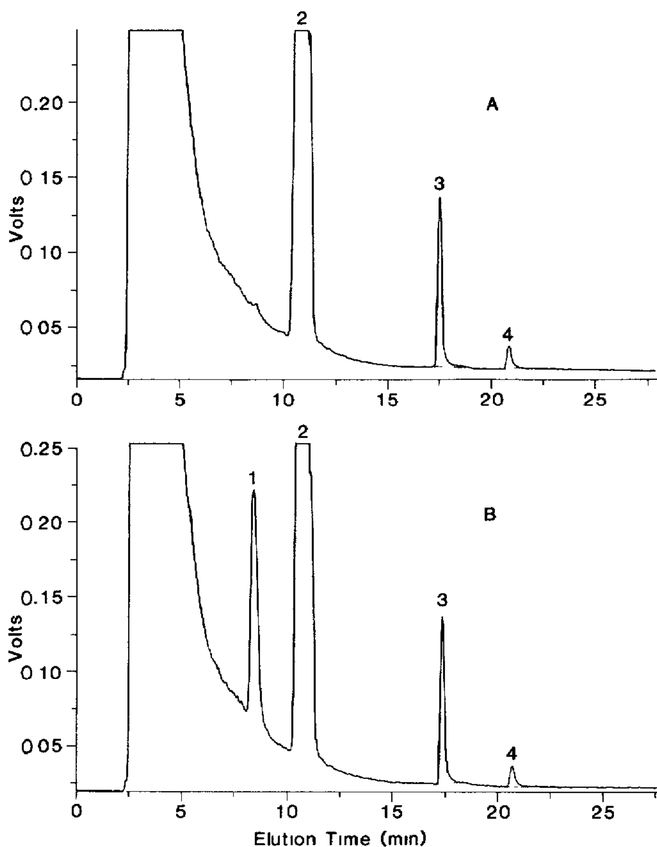

Fig. 1 HPLC separation of polyamines from a filtrate of a polyamine oxidase (PAO) assay on an Ultrasphere ion-pair column following post-column derivatization with *o*-phthalaldehyde and fluorescent detection using an excitation wavelength of 340 nm and an emission wavelength of 455 nm. A gradient elution technique was used with a sodium acetate–acetonitrile buffer (pH 4.5) supplemented with 10 m*M* octanesulfonic acid as the ion-pairing reagent as described in Experimental. The chromatograms shown were obtained using 50 μl of either (A) heat-denatured cytosol as an enzyme source for a blank PAO assay or (B) cytosol from supernatant of colonocyte homogenate obtained by centrifugation at 100 000 *g* for 60 min. Peaks 1 = putrescine, 2 = N^1-acetylspermidine, 3 = spermidine, 4 = spermine

With resetting of intitial conditions and column equilibration, this allowed for individual samples to be processed in 30 min.

Fig. 1A is a representative chromatogram from a blank PAO assay run with heat-denatured cytosol. The largest peak following the solvent front was identified as N^1-acetylspermidine (peak 2, Fig. 1A) both by its retention time compared to known standard as well as by its disappearance and conversion to spermidine following acid hydrolysis as previously described [20]. This N^1-acetylspermidine peak was entirely accounted for by the known concentration of this compound in the reaction mixture and there was no significant contribution from endogenous N^1-acetylspermidine in the cytosol used as enzyme source. Peaks 3 and 4, however, which were identified as spermidine and spermine, respectively, do represent contamination from endogenous polyamines present in the cytosol used as enzyme source. No peak corresponding to endogenous putrescine was detected in the blank run. In tissues with higher basal putrescine levels being assayed for PAO, such contaminating peaks in the blank would have to be corrected for by subtracting out this endogenous putrescine level.

Fig. 1B is a chromatogram of filtrate obtained after running a PAO assay in the presence of active enzyme using colonocyte cytosol as an enzyme source. In contrast to Fig 1A, a new peak has appeared (peak 1) which was identified as putrescine by its retention time. This was accompanied by a commensurate decrease in the N^1-acetylspermidine peak and no significant change in the spermidine or spermine peaks and is suggestive of PAO activity This finding is in

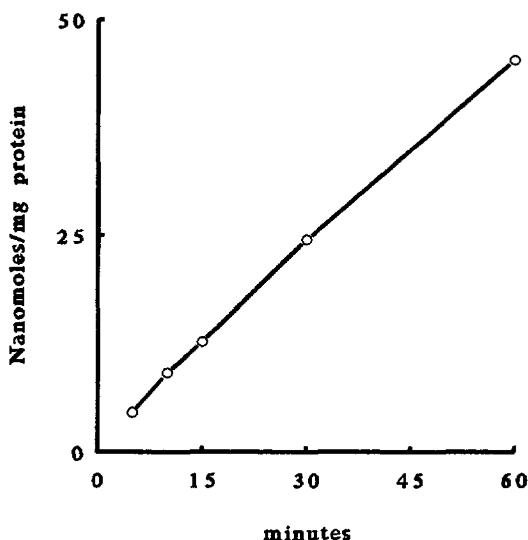

Fig 2. Polyamine oxidase activity expressed as nanomoles of putrescine formed per milligram of cytosolic protein as a funtion of time Amounts of putrescine formed were determined by HPLC analysis of a 50-μl aliquot of reaction mixture stopped at various times by addition of 50% trichloroacetic acid. Samples were filtered through a 0 45-μm filter and injected directly onto the column as described in Experimental

agreement with those of others who have reported that, although PAO has some activity towards spermidine and spermine, its preferred substates are the acetylated derivatives [8,9].

Time and protein dependence

Fig. 2 shows the results of the enzyme assay as a function of time and demonstrates that PAO activity was linear up to 60 min. At these later time points, however, there was significant depletion of substrate and therefore all subsequent assay were run for 15–30 min. Under these assay conditions the enzyme activity in nanomoles of putrescine formed per minute was found to be linear with respect to the amount of enzyme used, up to 500 μg of protein (data not shown).

PAO enzyme kinetics

By varying the substrate concentration from 10 to 500 μM, Lineweaver–Burk plots for PAO activity in the colon and small intestine were constructed. The calculated Michaelis constants were 27 and 36 μM for the colon and small intestine, respectively, which are in general agreement with reported K_M values for this enzyme [9]. The maximal velocity, V_{max}, for PAO was 39 nmol/mg/h in the colon and 65 nmol/mg/h in the small intestine. The PAO activities noted in these intestinal tissues are substantially higher than those originally reported by Hölttä [11], which is an expected finding in view of the fact this original method used spermidine as a substrate instead of its preferred, acetylated derivative used in the present studies.

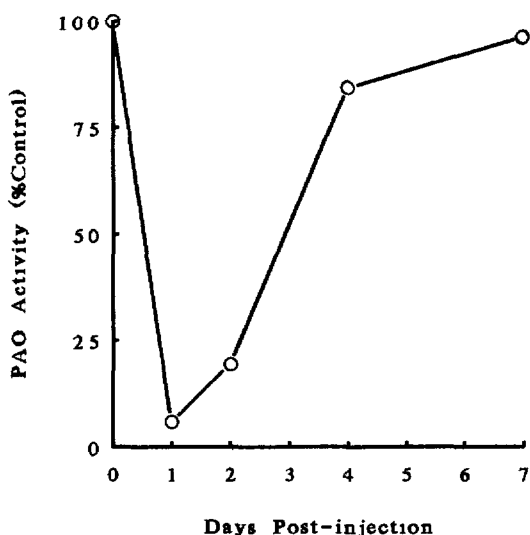

Fig 3 Inhibition of polyamine oxidase (PAO) activity as a function of time following intraperitoneal injection of either the PAO inhibitor MDL 72527 (20 mg/kg body weight) or saline PAO activity is expressed as a percent of control values and is based on $n = 4$ for each time point evaluated

PAO inhibition studies

A further confirmation that this assay technique truly measures PAO activity was obtained with the use of N^1,N^2-bis(2,3-butadienyl)-1,4-butanediamine (MDL 72527), a specific, enzyme-activated, irreversible inhibitor of PAO that has been studied extensively [21,22] Fig. 3 shows the results of colonic PAO activity one, two, four and seven days following intraperitoneal injection of MDL 72527 at a dose of 20 mg/kg body weight. PAO activity expressed as a percent of control is seen to fall substantially to less than 5% of control values 24 h after injection of this inhibitor followed by gradual recovery to 96% of control values by day 7. Because of the high specificity of MDL 72527 for inhibition of PAO [22], these results, in combination with the demonstrated saturation kinetics, and protein and time dependence would strongly suggest that this HPLC method is a valid technique for measuring PAO activity.

DISCUSSION

PAO is an important flavin-dependent peroxisomal enzyme which catalyzes the oxidative breakdown of N^1-acetylspermidine and N^1-acetylspermine to form putrescine and spermidine, respectively, releasing 3-acetamidopropanal and hydrogen peroxide in the process [12]. This reaction is preceded by acetylation of spermine and spermidine by the enzyme SAT which is the rate-limiting step in this reverse pathway [3,12]. In most tissues studied, PAO is constitutively expressed at high levels relative to SAT activity, thus maintaining low intracellular levels of the acetylated polyamines [23]. Although PAO does not appear to be an inducible enzyme [23], more recently others have found that significant changes in the activity of PAO can occur during tissue regeneration [24]. Thus, Hayashi *et al.* [24] found a three-fold increase in rat liver peroxisomal PAO activity during regeneration of the liver following partial hepatectomy and suggested that PAO may play an important role in regulating intracellular concentrations of polyamines.

Current methods for assaying PAO have several disadvantages including the requirements for radiolabelled substrates or detection methods which require lengthy derivatization procedures. Other methods, based upon measurement of reaction by-products, are difficult to interpret since these products can be formed by other oxidases in the cell or in other cases may directly affect PAO activity

The efficient separation of polyamines by HPLC and their sensitive detection by post-column fluorescent derivatization have allowed newer approaches to measuring PAO activity. This paper describes and validates an HPLC technique for measuring PAO activity using a reversed-phase, ion-pairing column which completely separates the substrate N^1-acetylspermidine from its reaction product, putrescine, in less than 15–30 min. In addition to the rapid turn-around time and use of the readily available and preferred substrate, N^1-acetylspermidine, this method does not require the use of radiolabelled substrates nor does it require any lengthy sample preparations or derivatization procedures.

The validity of this method is supported by our determination of the K_M values for intestinal PAO which are in agreement with those published previously using alternative methods [9]. Furthermore, the finding of nearly complete loss of PAO activity by our method in rats administered the specific PAO inhibitor N^1,N^2-bis (2,3-butadienyl)-1,4-butanediamine (MDL 72527) lends further support to this contention. This method therefore provides an accurate means for measuring PAO activity, an enzyme which plays a fundamental role in the regulation of polyamine metabolism

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