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Measurement of aspartate carbamoyltransferase activity by high performance liquid chromatography

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We developed an assay which permits measurement of aspartate carbamoyltransferase (ACTase) activity. Cytosol from human peripheral blood mononuclear cells was used as the enzyme source. Using [^{14}C]carbamoyl phosphate as the radiolabeled substrate, the formation of [^{14}C]carbamoyl aspartate was quantitated by high performance liquid chromatography (HPLC) using an anion-exchange column with UV detection at 200–280 nm and an on-line liquid scintillation detector. A gradient method from an initially low concentration of ammonium phosphate, 1 mM (pH 3.0), to a higher concentration, 38 mM (pH 4.5), was used. The apparent K_m values of carbamoyl phosphate and aspartate were 58 μM and 1.9 mM, respectively. ACTase inhibition by *N*-(phosphonacetyl)-l-aspartate (PALA) was consistent with a competitive model with respect to carbamoyl phosphate. The assay conditions were optimized to permit measurement of ACTase activity prior to and following therapy with PALA; ACTase was inhibited in a dose-dependent manner. This HPLC method permits direct quantitation of both the product of the reaction and the initial integrity of the substrate, [^{14}C]carbamoyl phosphate, which is unstable in aqueous solutions.

Key words: Aspartate carbamoyltransferase, biochemical monitoring, HPLC, *N*-(phosphonacetyl)-1-aspartate.

Introduction

Aspartate carbamoyltransferase (ACTase, EC 2.1.3.2) catalyzes the second step in *de novo* pyrimidine biosynthesis: the formation of carbamoyl aspartate from carbamoyl phosphate and aspartate (Figure 1). *N*-(phosphonacetyl)-L-aspartate (PALA), a transition state analog inhibitor of ACTase, is currently undergoing clinical evaluation as a biochemical modulator of the antimetabolite 5-fluorouracil in the treatment of patients with gastrointestinal malignancies.[1,2] Inhibition of ACTase by PALA results in a decrease in uridine and cytidine nucleotide pools, and accumulation of phosphoribosylpyrophosphate.[2] Preclinical studies suggest that PALA may enhance the metabolism of fluorouracil and increase its cytotoxicity through both RNA- and DNA-directed mechanisms. In addition to evaluating the impact of PALA on the clinical toxicity of fluorouracil, determination of ACTase activity in patient samples pretreatment and following PALA therapy would provide critical information to aid selection of an appropriate 'modulatory' dose of PALA.

Several methods to measure ACTase activity have previously been reported. Carbamoyl phosphate is unstable in aqueous medium and, as a phosphorylated compound, it cannot readily enter cells; thus, all reported methods of measuring ACTase activity have used cell homogenates. Porter used uniformly labeled [U-^{14}C]aspartate, which required purification by adsorption and elution from Dowex 50-8X (H^+ form) cation exchange resin.[3] After incubation of purified [U-^{14}C]aspartate

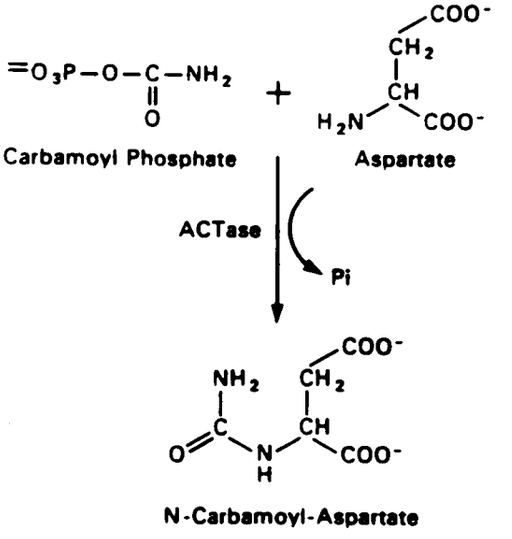

Figure 1. Production of carbamoyl aspartate from carbamoyl phosphate and aspartate by ACTase.

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with carbamoyl phosphate and enzyme, [^{14}C]carbamoyl aspartate was separated from unreacted [U-^{14}C]aspartate by differential adsorption and elution from Dowex 50 (H^+ form). Aspartate binds through its α-amino group to the cation exchange resin; carbamoyl aspartate elutes with water, while elution of aspartate requires acid. A limitation of this assay is the assumption that all radioactive counts eluting with water represent carbamoyl aspartate. Alternatively, Jayaram and colleagues used custom-synthesized aspartate labeled at the fourth carbon position.[4] Enzymatic decarboxylation was required to release $^{14}CO_2$ from unreacted [4-^{14}C]aspartate; [4-^{14}C]carbamoyl aspartate was unaffected by the decarboxylation step. Decarboxylation required several steps: after the initial incubation, excess α-ketoglutarate was added to the reaction mixture. The addition of glutamate oxaloacetate transaminase then catalyzed the synthesis of [^{14}C]oxaloacetate and glutamate from unreacted [4-^{14}C]aspartate. Excess zinc ions were then added, resulting in the chemical degradation of oxaloacetate to pyruvate and $^{14}CO_2$. Dissipation of radiolabeled CO_2 was then achieved by incubating open vessels in a hood. The residual ^{14}C counts were presumed to represent [^{14}C]carbamoyl aspartate. A disadvantage of this method is that incomplete decarboxylation of unreacted substrate would lead to overestimation of catalytic activity; further, this uniquely labeled isotope is no longer commercially available. While aspartate serves as a substrate in numerous enzymatic reactions, carbamoyl phosphate is a substrate only for ACTase and ornithine carbamoyltransferase. Several investigators have published assays using [^{14}C]carbamoyl phosphate as the substrate for determination of ACTase activity.[5,6] Davies *et al.* employed a 1 h incubation, following which an aliquot of the reaction mixture was pipetted into 0.2 M acetic acid; the samples were then evaporated to dryness at 80°C to eliminate the degradation product ($^{14}CO_2$) of unreacted carbamoyl phosphate. The residual acid-stable radioactivity was presumed to represent [^{14}C]carbamoyl aspartate. While the use of [^{14}C]carbamoyl phosphate with this method overcomes some of the problems associated with the use of [^{14}C]aspartate, it does not permit quantification of the amount of non-degraded carbamoyl phosphate at the start of the reaction; in addition, incomplete hydrolysis of unreacted substrate might lead to overestimation of product formation.

Previously published assays which attempted to measure inhibition of PALA in patient samples used very high concentrations of carbamoyl phosphate (1 or 4 mM).[7,8] Further, the tissue or cell samples were disrupted in a large volume of buffer relative to the amount of cytosol used in the reaction mixture, such that only a small proportion of the cytosol was ultimately used, and it constituted only 17–25% of the volume in the final assay mixture.[7,8] Because inhibition of ACTase by PALA is competitive with respect to carbamoyl phosphate, use of excess substrate might diminish the opportunity to detect a PALA effect. Dilutional effects resulting from use of a small percentage of the cytosol relative to the total assay volume might also lead to underestimation of the effect of PALA on ACTase. Finally, there is no convenient way to document the integrity of non-radiolabeled carbamoyl phosphate. Since carbamoyl phosphate is unstable in aqueous solutions, undetected degradation might lead to variability and errors in substrate concentration, which would be especially problematic when a low carbamoyl phosphate concentration is necessary. For these reasons, we developed a direct high performance liquid chromatography (HPLC) assay for ACTase activity which could document the integrity of the substrate prior to use, and distinguish unreacted [^{14}C]carbamoyl phosphate from radiolabeled carbamoyl aspartate and any possible distal metabolites [dihydroorate (DHO), orotic acid, orotidine or orotidine monophosphate] formed during the assay. We employed a very brief incubation period in an effort to limit the chemical hydrolysis of [^{14}C]carbamoyl phosphate in aqueous medium, and optimized the assay conditions to enhance the ability to detect inhibition of ACTase in peripheral blood mononuclear cells at baseline and following PALA administration in patient samples.

Materials and methods

Materials

[^{14}C]Carbamoyl phosphate, dilithium salt (17.6 mCi/mmol), was obtained as a crystalline solid, 98% pure, from NEN Research Products (DuPont, Wilmington, DE), and was stored at −70°C. Immediately prior to daily use, an aliquot of the crystalline solid was diluted in buffer (50 mM Tris–HCl, pH 8.0) and placed on ice; the radioactivity was determined, and the aqueous solution was frozen on dry ice until it was added to the reaction mixture. The integrity of [^{14}C]carbamoyl phosphate was documented by HPLC analysis (described below). [4-^{14}C]Aspartate (54 mCi/mmol)

was obtained from Amersham (Arlington Heights, IL). Bacterial ACTase (*Escherichia coli*), carbamoyl phosphate, carbamoyl aspartate, DHO, orotic acid, orotidine and all nucleotides were obtained from Sigma (St Louis, MO). PALA was provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, National Cancer Institute. Lymphocyte Separation Medium™ (LSM) was obtained from Organon Teknika (Durham, NC). Phosphate-buffered saline (PBS 1×, pH 7.4) was obtained from Biofluids (Rockville, MD). Gelman Acrodisc® filters (LC13 PVDF, 0.45 μm) were obtained from PGC Scientifics (Gaithersburg, MD).

Patient population

Peripheral blood was obtained from either normal laboratory volunteers or from consenting patients enrolled on The National Institutes of Health Clinical Center Protocol 89-C-170, "A Phase I study of continuous infusion of 5-fluorouracil plus calcium leucovorin in combination with PALA in metastatic gastrointestinal carcinoma". Separate patient cohorts received escalating doses of PALA ranging from 0.25 to 2.9 g/m^2 (i.v.) over 30 min, 24 h prior to the start of a 72 h infusion of fluorouracil/leucovorin. Blood samples (10 ml) were taken immediately prior to the dose of PALA and again 24 h later, prior to the start of fluorouracil/leucovorin.

Isolation of peripheral blood mononuclear cells and erythrocytes

A modification of the standard ficoll–sodium diatrizoate density separation technique was used.[9] Briefly, 10 ml of heparinized peripheral blood was diluted with 25 ml PBS; 15 ml LSM™ was carefully pipetted below the diluted blood as an underlayer, then the tube was centrifuged at room temp for 30 minutes at 800 × ***g***. During centrifugation, differential migration results in the formation of several layers: both erythrocytes and granulocytes migrate through the gradient. Lymphocytes, monocytes and platelets are recovered by aspirating the mononuclear cell layer at the plasma/LSM™ interface; further washing with 50 ml PBS followed by low speed centrifugation as above separates platelets, LSM™ and plasma from the pellet containing the lymphocytes and monocytes. The purified lymphocytes/monocytes, which we will refer to as peripheral blood mononuclear cells, were then suspended in 1 ml of PBS; an aliquot was subjected to red blood cell lysis with one drop of ZAP® (potassium cyanide; Sigma) and counted by hemacytometer. The remaining cells were then centrifuged at 400 × ***g*** at 4°C in a refrigerated microfuge (Juoan, Winchester, VA), excess PBS was gently aspirated and the cell pellet was frozen at −70°C until analysis. Erythrocytes were isolated from the bottom of the cell pellet after ficoll–sodium diatrizoate centrifugation; after washing, the cell pellet was frozen at −70°C until analysis. Protein was measured by the method of Bradford.[10]

Cytosolic assay for ACTase

We initially evaluated ACTase activity using a modification of a previously described method which used [4-^{14}C]aspartic acid (Asp) (54 mCi/mmol).[8,11,12] The mononuclear cell pellet was reconstituted in 100 μl buffer containing 30% dimethylsulfoxide, 5% glycerin, 0.1 M Tris–HCl (pH 8.4), 0.1 M KCl, 0.2 mM EDTA and 2 mM dithiothreitol. The cells were disrupted by sonication using 4 × 1 sec pulses on setting 25 with a VibraCell™ sonicator (Sonics and Materials, Danbury, CT) equipped with a Teledyne probe; the supernatant was isolated after centrifugation. In preliminary studies performed to optimize the assay conditions, the concentration of carbamoyl phosphate and aspartate varied. The conditions selected for the patient samples were as follows: the final assay volume was 40 μl and contained 10 μl cytosol, 210 μM L-[4-^{14}C]aspartate (10^6 d.p.m.) and 30 μM carbamoyl phosphate in 50 mM Tris–HCl buffer (pH 8.5). The reactants were incubated in a 37°C shaking water bath for 10 min; the reaction was stopped by boiling for 2 min. The excess L-[4-^{14}C]aspartate was dissipated by the addition of 50 μl of a decarboxylation reagent as previously described.[8,11,12] The vials were immersed in a liquid scintillation vial containing 10 ml of liquid scintillant. The residual ^{14}C, presumed to represent [^{14}C]carbamoyl aspartate, was quantitated in a liquid scintillation counter. Paired samples were considered evaluable if the radioactivity in the baseline sample for each patient cycle was more than 2-fold above background (which averaged 13193 ± 1688 d.p.m.). Baseline activity in 55 samples was 2-fold or greater above background in only 22 (40%). ACTase activity was expressed as pmol [^{14}C]carbamoyl aspartate formed/min/10^6 nucleated cells.

Because of the problems with high-background counts with the [4-^{14}C]aspartate assay, we developed a HPLC assay using [^{14}C]carbamoyl phosphate as the substrate. The source of the ACTase for the kinetic studies was peripheral blood mononuclear cells isolated from 50 ml blood from normal volunteers; the peripheral blood mononuclear cells were disrupted by sonication in 400 μl of 50 mM Tris-HCl (pH 8.0) and centrifuged at 12 000 × *g* for 15 min; 10 μl of supernatant was used in a total reaction volume of 50 μl. Erythrocyte ACTase was obtained from the erythrocyte pellet; after sonication, 60 μl of a 1 ml sample was used in a final reaction volume of 80 μl. The aspartate stock was prepared in 200 mM Tris–HCl, pH 8.0, such that upon dilution the final buffer concentration in the assay was 60 mM. The final concentration of aspartate ranged from 125 μM to 5 mM in the various experiments. The concentration of carbamoyl phosphate was varied by adjusting the amount of radioactive label used. The d.p.m. in the assay mixture (final volume = 50 μl) ranged from 4000 to 770 000, corresponding to 2–400 μM carbamoyl phosphate (17.6 mCi/mmol). Alternatively, purified bacterial ACTase was used as the enzyme source.

In preliminary experiments, replicate samples were incubated for various times from 5 to 60 min. The reaction was quenched by adding 500 μl 1 mM ammonium phosphate, pH 3.0 (the initial mobile phase) followed by immediate placement of the tubes on dry ice. The samples were then frozen at −70°C until analysis by HPLC. The ability of PALA to inhibit the ACTase reaction was determined by incubating replicate samples with PALA at concentrations ranging from 1 nM to 1 mM.

To measure ACTase activity in peripheral blood mononuclear cells isolated from patients at baseline and following PALA, the cell pellet was reconstituted in 50 μl 50 mM Tris–HCl (pH 8.0); after sonication, 30 μl of supernatant was mixed in a total assay volume of 40 μl containing 2 mM aspartate and [^{14}C]carbamoyl phosphate (17.6 mCi/mmol) at a final concentration of 50 μM. Thus, the cytosol comprised 75% of the final assay volume. The average proportion of cytosol used in the assay for the paired baseline and 24 h samples was identical, 50%. The assay proceeded as described above except that the duration of incubation at 37°C ranged from 2 to 10 min. The reaction was linear over the range of 200–1600 pmol carbamoyl aspartate formed. Data from the linear portion of the reaction were used to calculate ACTase activity. The reaction was linear for 10 min using 6 million cells or less; with higher cell numbers, data from shorter incubation times (2–5 min) were used.

Enzyme kinetics were determined by a software program entitled ENZYME-PC, a computer analysis of enzyme–substrate–inhibitor kinetic data, developed by R. A. Lutz and D. Rodbard (National Institute of Child Health and Human Development, Bethesda, MD).

HPLC assay

The Waters (Waters Chromatography Division, Millipore, Bedford, MA) analytical HPLC system included a 600E system controller, a 990 photodiode array detector, an NEC Powermate 2 APC-IV computer with Powerline™ software and a U6K injector. A Flo-One Beta liquid scintillation flow detector (Radiomatic, Tampa, FL) was on-line. A SAX Radial-Pak column (Waters) was developed using two buffers as the mobile phase: buffer A was 0.001 M ammonium phosphate, pH 3.0, and buffer B was 0.375 M ammonium phosphate, pH 4.5. The flow rate was 2 ml/min. An isocratic gradient of 100% buffer A was run for 3 min, followed by a linear gradient to 96% buffer A/4% buffer B over 25 min, then to 90% A/10% B over 15 min. Isocratic elution at 90% A/10% B was continued for 20 min, followed by a linear gradient to 85% A/15% B over 15 min. The column was washed with 100% B for 4 min and then allowed to equilibrate for 15 min at initial conditions prior to the next run. This method will be referred to as HPLC method 1. An aliquot of sample was filtered through a Gelman Acrodisc and injected along with cold standards into the HPLC system. UV absorbance was monitored between 200 and 280 nM; DHO and carbamoyl aspartate standards were detected at 200–210 nM; the remaining pyrimidine nucleosides and nucleotides were detected at both 200–210 and 260 nM. Carbamoyl phosphate is non-UV absorbing at 200–280 nm; its retention time was identified using [^{14}C]carbamoyl phosphate standard immediately upon its reconstitution in aqueous solution. The retention times were as follows: DHO, orotic acid, orotidine, 27 min; carbamoyl aspartate, 44 min; carbamoyl phosphate, 58 min; orotidine monophosphate, 62 min; UDP, 69 min.

^{14}C-labeled nucleotides (orotidine monophosphate, UMP or UDP) were not detected under the conditions described for this assay in 29 separate determinations, presumably because the endogenous ATP concentration was insufficient to promote detectable nucleotide formation. There-

after, an abbreviated HPLC method (termed HPLC method 2) was developed which could separate carbamoyl phosphate, carbamoyl aspartate and DHO/orotate/orotidine. An isocratic gradient of 100% buffer A was run at 2 ml/min for 3 min; the following linear gradients were then run: to 90% A/10% B over 17 min; to 80 A/20% buffer B over 10 min; to 70%A/30%B over 8 min, then to 100% B over 4 min. The column was then allowed to equilibrate for 10 min with initial conditions before the next run. The retention times were as follows: DHO/orotate/orotidine, 14 min; carbamoyl aspartate, 27 min; carbamoyl phosphate/orotidine monophosphate, 36 min.

The amount of carbamoyl aspartate formed was quantitated by multiplying the total amount of carbamoyl phosphate added to the reaction mixture by the percent of radioactive counts coeluting with carbamoyl aspartate and dihydroorotate standards. Enzyme activity was expressed either as pmol metabolites formed/min/10^6 nucleated cells or as pmol/minute/mg protein.

Results

ACTase activity in cytosol isolated from peripheral blood mononuclear cells was initially measured according to a previously published method using [4-^{14}C]aspartate as the substrate.[8,11,12] Because PALA is a competitive inhibitor of ACTase with carbamoyl phosphate as the variable substrate, we wished to use non-saturating concentrations of carbamoyl phosphate to increase the ability to detect inhibition of the reaction by PALA in paired patient samples. We selected 30 μM carbamoyl phosphate as the substrate concentration for the additional studies based upon data (K_m) from the literature. A series of experiments were conducted to optimize the conditions of the assay for use in patients receiving PALA. Optimal results were obtained using undiluted L-[4-^{14}C]aspartate (54 mCi/mmol; 1×10^6 d.p.m. per sample gave a final concentration of 210 μM). After terminating the reaction by boiling, residual ^{14}C was measured following enzymatic decarboxylation to release $^{14}CO_2$ from unreacted substrate.

Peripheral blood mononuclear cells were isolated from patients immediately prior to and 24 h following 0.25 g/m^2 PALA. The usefulness of the 4-^{14}C assay was limited by high and variable background counts. Among 55 determinations of pretreatment enzyme activity, the residual radioactivity after decarboxylation, presumed to represent [^{14}C]carbamoyl aspartate, was 2-fold or greater above background in only 40%. Using only data from 15 paired samples in which baseline residual counts were 2-fold or greater than background, ACTase activity from eight patients treated with 0.25 g/m^2 PALA averaged 14.3 pmol/min/10^6 cells (Table 1). At 24 h, ACTase activity decreased by an average of 29%; the degree of inhibition was 50% or greater in only five treatment cycles (33%).

The high background counts relative to the residual radioactivity in baseline patient samples posed a major problem. Further, during the conduct of these experiments, the [4-^{14}C]aspartate became unavailable commercially. We therefore developed a new HPLC assay; [^{14}C]carbamoyl phosphate was selected as the radiolabeled substrate because of its relative specificity for the ACTase reaction. The retention times of radiolabeled and non-radiolabeled standards were initially determined by anion exchange HPLC method 1. After incubation of the reaction mixture in the absence of an enzyme source, only two peaks derived from

Table 1. Effect of PALA administration on ACTase activity in peripheral blood mononuclear cells

	PALA (g/m^2)			
	0.25	0.25	1.3	2.8
Assay	[4-^{14}C]Asp	[^{14}C]CP	[^{14}C]CP	[^{14}C]CP
No. cycles	15	6	23	5
(No. patients)	(8)	(3)	(13)	(3)
ACTase activity (mean ± SE)				
0 h	14.3 ± 2.5	39.0 ± 10.8	32.2 ± 3.2	43.3 ± 5.4
24 h	10.2 ± 2.5	41.0 ± 16.3	17.6 ± 2.8	12.3 ± 2.1
(% 0 h)	(71%)	(105%)	(55%)	(28%)
Cycles with ≥50% inhibition at 24 h	33%	17%	65%	100%

carbamoyl phosphate were identified: the majority (over 90%) of the counts eluted at the same retention time as [^{14}C]carbamoyl phosphate standard. The balance (10%) eluted at 3 min presumably represented products of hydrolysis.

Incubation of the reaction mixture with cytosol resulted in the appearance of a new ^{14}C peak co-eluting with carbamoyl aspartate standard at 44 minutes. [^{14}C]Carbamoyl aspartate was readily separated from the substrate (eluting at 58 min), as well as distal metabolites (identified by injection of standards) by HPLC method 1. As a further validation, the reaction mixture containing [^{14}C]carbamoyl phosphate, cold aspartate and 50 mM Tris–HCl buffer (pH 8.0) was incubated with purified *E. coli* ACTase. With bacterial ACTase, three peaks were seen: carbamoyl phosphate, carbamoyl aspartate and the hydrolysis product, similar to that observed after incubation with cytosolic enzyme. Further, the production of the putative [^{14}C]carbamoyl aspartate peak was inhibited by PALA in a dose-dependent manner.

Since stability of the substrates and products are critical to accurate quantitation, we measured the stability of carbamoyl aspartate, carbamoyl phosphate and ACTase in cytosol isolated from peripheral blood mononuclear cells. The stability of [^{14}C]carbamoyl aspartate was initially determined after incubation of [^{14}C]carbamoyl phosphate (33 μM) in the reaction buffer (containing 125 μM cold aspartate) with or without cytosol at 37°C for 60 min (to allow the reaction to go to completion). The reaction was then quenched with excess cold 1 mM ammonium phosphate, pH 3.0, and the samples were then placed on ice. An aliquot from the sample was analyzed immediately by HPLC and again after the sample had been on ice for 6 h. In the sample containing cytosol, [^{14}C]carbamoyl aspartate accounted for 64 and 63% of the metabolites, respectively, before and after a 6 h incubation on ice, indicating stability of carbamoyl aspartate. The stability of [^{14}C]carbamoyl phosphate was determined by comparing the distribution of radioactivity by HPLC analysis immediately after reconstituting the isotope in 50 mM Tris–HCl (pH 8.0), and again after a 1 or 6 h incubation on ice or at 37°C as described above. Parent compound accounted for 95% of the ^{14}C counts immediately upon reconstitution. After 1 and 6 h incubations at 37°C, however, [^{14}C]carbamoyl phosphate comprised 81 and 34% of the counts, respectively. The rate of degradation was slower if [^{14}C]carbamoyl phosphate was kept on ice: after 6 h, 84% of parent compound remained. However degradation of [^{14}C]carbamoyl phosphate also occurred, even if the aqueous sample was stored at or below −20°C.

Enzyme activity appeared to be well preserved for prolonged periods provided the intact cell pellet was stored at −70°C. However, disruption of the cells by sonication followed by freezing and storage of the cytosol at −20°C overnight resulted in an average loss of 53 ± 14% of ACTase activity (n = 6) compared with that obtained with freshly prepared cytosol.

Microscopic inspection of the peripheral blood mononuclear cells isolated by centrifugation in LSM™ revealed contamination by red blood cells. Protein concentrations in cytosolic extracts from red blood cells and the lymphocytes/monocytes differed by several orders of magnitude, presumably because of the much higher numbers of circulating erythrocytes compared to lymphocytes. In paired samples from two patients, the protein content averaged 9.2 mg/ml in peripheral blood mononuclear cells compared with 345 mg/ml in erythrocytes. ACTase activity was 318-fold lower, however, in erythrocytes (average of two samples): 2.0 pmol/minute/mg protein compared with 635 pmol/minute/mg protein in peripheral blood mononuclear cells. These observations suggest that expression of ACTase activity as amount of carbamoyl aspartate formed per minute per 10^6 nucleated cells would be less subject to error than if protein concentration was used.

We wished to determine inhibition of ACTase activity by PALA using several different combinations of aspartate and carbamoyl phosphate concentrations (Figure 2). Absolute ACTase activity in the absence of inhibitor increased as the concentration of substrate increased: with either 1 or 5 mM aspartate, ACTase activity increased 1.8-fold when the concentration of carbamoyl phosphate increased from 28 to 168 μM. PALA inhibited ACTase activity in a dose-dependent manner. With 28 μM [^{14}C]carbamoyl phosphate and 1 mM aspartate as the substrates, the IC_{50} of PALA was 6 nM. When the concentration of [^{14}C]carbamoyl phosphate was increased 6-fold, the IC_{50} increased about 8-fold to 50 nM. Comparison of the IC_{50} values for PALA with the carbamoyl phosphate concentration suggests that its affinity for ACTase is three orders of magnitude greater than that of carbamoyl phosphate. Increasing the concentration of aspartate 5-fold to 5 mM had no effect on the IC_{50} of PALA with either [^{14}C]carbamoyl phosphate concentration. These data are consistent with competitive inhibition of ACTase activity by PALA with respect to carbamoyl

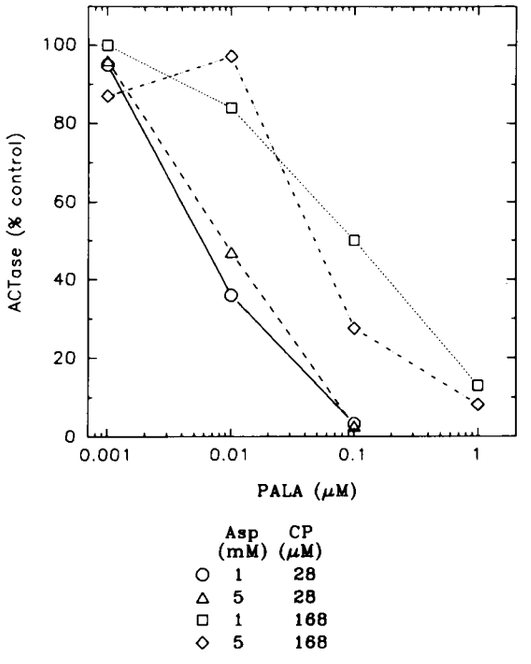

Figure 2. Dose-dependent inhibition of ACTase by PALA. Cytosol from peripheral blood mononuclear cells of a normal volunteer was incubated with either 1 or 5 mM aspartate and either 28 or 168 μM [^{14}C]carbamoyl phosphate in the presence or absence of PALA at the indicated concentrations for 10 min at 37°C. ACTase activity was determined by HPLC analysis. The amount of cytosol used for each condition was taken from the equivalent of 420 000 cells. The data are presented as ACTase activity (percent of control) versus PALA concentration. Absolute ACTase activity (pmol/minute/10^6 cells) for the control sample of each condition is as follows: 1 mM aspartate/28 μM carbamoyl phosphate, 91; 5 mM aspartate/28 μM carbamoyl phosphate, 157; 1 mM aspartate/168 μM carbamoyl phosphate, 132; 5 mM aspartate/168 μM carbamoyl phosphate, 284.

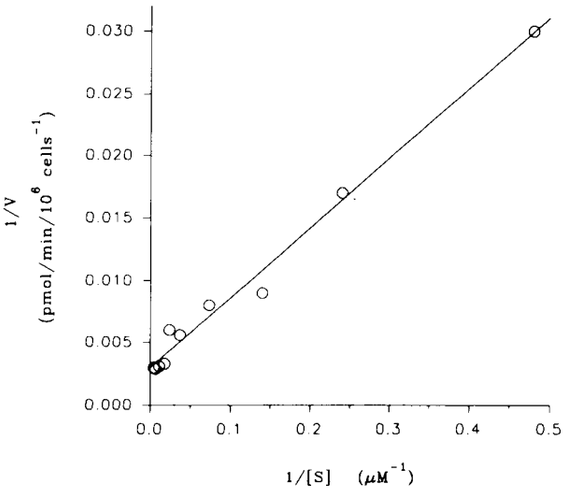

Figure 3. Kinetic studies of ACTase with [^{14}C]carbamoyl phosphate as the variable substrate. Cytosol from peripheral blood mononuclear cells of a normal volunteer was incubated at 37°C with 5 mM aspartate and concentrations of [^{14}C]carbamoyl phosphate ranging from 2 to 225 μM in a total volume of 50 μl. The amount of cytosol used for each condition was taken from the equivalent of 390 000 cells. Aliquots from each condition were taken at 5, 10 and 15 min. ACTase activity was determined by HPLC method 1 as described in the Methods. A Lineweaver–Burke plot is shown which depicts the inverse of the reaction velocity as a function of the inverse of substrate concentration. In this experiment, the maximal velocity (V_{max}) was 369 pmol/min/10^6 cells and the K_m was 23 μM.

phosphate, and non-competitive inhibition with respect to aspartate as previously reported.[7,8,11–18]

The K_m of carbamoyl phosphate for ACTase isolated from peripheral blood mononuclear cells was determined using varying [^{14}C]carbamoyl phosphate concentrations with a fixed aspartate concentration of 5 mM. A representative Lineweaver–Burke plot from a single determination is shown in Figure 3. The average K_m and maximum velocity for five separate experiments using peripheral blood mononuclear cells from different sources were (mean ± SEM): 59 ± 13 μM (range 23–108 μM) and 510 ± 130 pmol/min/10^6 cells (range 205–966 pmol/min/10^6 cells), respectively. Using excess carbamoyl phosphate (final concentration, 4 mM, 1.5 μCi/μmol), the K_m of aspartate was determined to be 1.9 mM.

We developed this HPLC assay to permit quantitation of ACTase activity in peripheral blood mononuclear cells isolated from patients receiving PALA on clinical trials. Because PALA is a competitive inhibitor of ACTase with respect to carbamoyl phosphate, we felt that it was important to use non-saturating concentrations of carbamoyl phosphate in the assay mixture. We reasoned that the concentration of carbamoyl phosphate should be low enough to maximize the assay sensitivity to PALA inhibition, yet be in the concentration range likely to be found intracellularly. We therefore selected concentrations of carbamoyl phosphate (50 μM) and aspartate (2 mM) approximating their K_m values.

The samples were incubated at 37°C and aliquots were taken at intervals from 2 to 10 min. Carbamoyl aspartate was the major metabolite formed and ^{14}C co-eluting with DHO accounted for less than 5% of the total metabolites. ACTase activity was

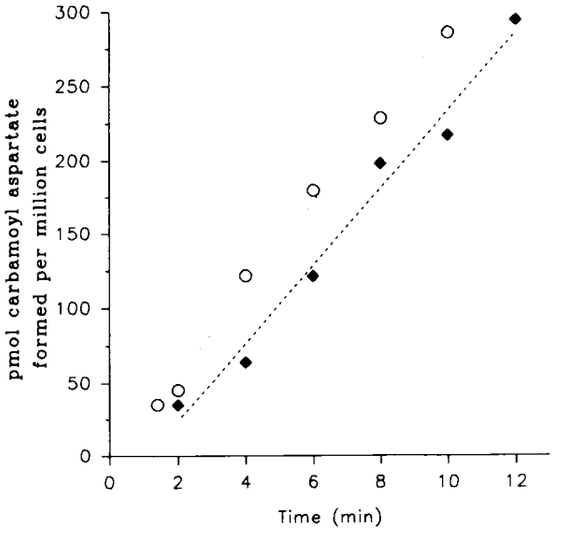

Figure 4. Time-course of [^{14}C]carbamoyl aspartate formation using patient samples. Baseline cytosol samples from peripheral blood mononuclear cells of patients were incubated in reaction mixture containing 30 μl cytosol, 2 nmol [^{14}C]carbamoyl phosphate, 80 nmol aspartate and 2.5 μmol Tris–HCl (pH 8.0) in a total volume of 40 μl. The following cell numbers were used: FN, 5.8 million cells; JN, 3.7 million cells. ACTase activity was determined by HPLC method 2. (◆) JN, (○) FN.

determined from the linear portion of the amount of product formed versus time curve. The reaction was linear for up to 10 min when the cell number was 6×10^6 or less (Figure 4). When the sample contained cytosol isolated from higher cell numbers, ACTase activity was calculated from the amount of [^{14}C]carbamoyl aspartate formed during shorter incubations (2–5 min).

In 36 cancer patients (median age 57 years, range 37–78 years) entered on our phase I trial of PALA in combination with 5-fluouracil and leucovorin, the median baseline ACTase activity was 40 pmol/min/10^6 cells (range 15–96). There was considerable interpatient variability and ACTase activity did not appear to be normally distributed (data not shown). The median enzyme activity was lower than that determined under similar conditions using peripheral blood mononuclear cells isolated from healthy and younger volunteers (median age 36 years, range 26–46 years): median 108 pmol/min/10^6 cells (range 43–242; $n = 15$). This interpatient variability highlights the importance of comparing the post-PALA ACTase activity to each patient's baseline value for each cycle. It was not possible to obtain all the patient samples at the same time of day. Blood samples were drawn between 9:00 a.m. and 3:00 p.m.; however, all blood samples within a given patient cycle (baseline and 24 h) were drawn at the same time of day. All matched samples for a given cycle were then assayed at the same time. In 62 paired baseline and 24 h samples, the average cell number used in the assay, 8.5×10^6, was similar at both time points.

Table 1 shows ACTase activity immediately prior to and 24 h following a single dose of PALA at either 0.25, 1.3 or 2.8 g/m^2. The higher baseline ACTase activity as determined with the new assay compared with the [4-^{14}C]aspartate assay is most likely due to the differences in substrate concentrations. With either assay, ACTase activity was not appreciably inhibited 24 h after administration of 0.25 g/m^2 PALA. In contrast, 24 h after administration of 1.3 and 2.8 g/m^2 PALA, the average ACTase activity was 55 and 28% of baseline. If the data for patients receiving 0.25 g/m^2 PALA using both ACTase assays is combined, ACTase activity at 24 h was inhibited by more than 50% in only six of 21 patient cycles (29%). With the intermediate and highest doses of PALA, however, ACTase activity at 24 h was inhibited by at least 50% in 65 and 100% of patient cycles, respectively.

Discussion

We developed an anion-exchange HPLC assay to measure substrate integrity as well as to quantitate the products of the ACTase reaction and optimize conditions to permit detection of the biochemical effects of PALA in patient samples. Because inhibition of ACTase by PALA is competitive with respect to carbamoyl phosphate, the concentration of carbamoyl phosphate used in the assay mixture can have a profound impact on the ability of PALA to interfere with the enzyme reaction. A reasonable option would be to use the concentration of carbamoyl phosphate predicted to occur in normal tissues. The concentration of carbamoyl phosphate in intact cells is probably low; however, it has proven difficult to quantitate because of the instability of the compound in aqueous medium.[9,10] Following incubation of [^{14}C]carbamoyl phosphate at 37°C, pH 8, we observed that degradation occurred at a rate of 14% per hour. Further, ACTase exists as a multi-enzyme complex with the first three enzymes of *de novo* pyrimidine biosynthesis: carbamoyl phosphate synthetase, ACTase and DHO.[13–16] ACTase not only has a very low K_m for carbamoyl phosphate, but its activity is much greater (300-fold) than that of carbamoyl phosphate

synthetase.[13,16] Thus, newly synthesized carbamoyl phosphate is immediately channeled to ACTase. We therefore elected to use a concentration of carbamoyl phosphate at its apparent K_m as determined under the conditions of this assay.

A comparison of the K_m of carbamoyl phosphate and the K_i of PALA for ACTase from various mammalian tissues or cell lines is shown in Table 2. The K_m of carbamoyl phosphate ranged from 0.5 to 58 μM with the various assays, and the apparent K_i of PALA ranged from 0.4 and 26 nM. Shoaf *et al.* reported that the pH optimum for ACTase was between pH 9.4 and 9.75; above that pH, the velocity of ACTase dropped rapidly.[15] A broad plateau in the rate of ACTase (about half of the maximal velocity) was noted at pH values between 8 and 9. We selected pH 8 to minimize the rate of base-catalyzed hydrolysis of carbamoyl phosphate.

While a number of studies have been published which describe inhibition of ACTase by PALA in cytosolic preparations of cancer cell lines or murine tumors, only limited information is available concerning the change in ACTase activity from patient samples following PALA administration, particularly after lower PALA doses. Moore *et al.* reported the change in ACTase activity in serial tumor biopsies taken from 16 patients at baseline and again at times ranging from 1.5 to 145 h following PALA (administered on one of several schedules).[7] Baseline ACTase activity ranged from 0.5 to 1.8 nmol/min/mg. At various times following administration of 1 g/m^2 PALA on a daily schedule lasting 8 days, ACTase was inhibited by 17–65%. ACTase was inhibited by 56–87% at 3–24 h following a single dose of 5 or 6 g/m^2 PALA. Because the concentration of carbamoyl phosphate was 1 mM (aspartate = 4 mM), the degree of ACTase inhibition might have been underestimated. Kensler *et al.* reported 80% inhibition of ACTase in peripheral blood leukocytes in a patient receiving 1500 mg/m^2 PALA on a daily schedule lasting 5 days. After cessation of therapy, ACTase activity was inhibited by 50% or more for up to 11 days after the fifth dose. The concentration of carbamoyl phosphate used in those studies, 4.2 mM, was very high.[8]

With our HPLC assay using [^{14}C]carbamoyl phosphate, we found that the IC_{50} of PALA was 6–8 nM with carbamoyl phosphate at about one-half of the K_m; increasing the concentration of carbamoyl phosphate to three times the K_m increased the IC_{50} of PALA to 50–100 nM. We selected 50 μM carbamoyl phosphate for the ACTase assay using peripheral blood mononuclear cells isolated from patients. Dose-dependent inhibition of ACTase activity was noted and the enzyme was inhibited by over 70% at 24 h following administration of 2.9 g/m^2 of PALA.

In contrast to previously published assays which generally employed 1–4 mM carbamoyl phosphate, well in excess of its K_m, we specifically wished to use non-saturating concentrations of [^{14}C]carbamoyl phosphate to increase the sensitivity of detecting inhibition of the reaction by PALA. We felt that documentation of the integrity of [^{14}C]carbamoyl phosphate was of crucial importance for two reasons: it is inherently unstable in aqueous solutions and its concentration profoundly influences the ability to detect inhibition by PALA. The instability of the compound, coupled with the relative inability of carbamoyl phosphate to

Table 2. Properties of mammalian ACTase in various assays

Reference	Enzyme source	[^{14}C]Substrate	Method	K_m carbamoyl phosphate (μM)	K_m aspartate (mM)	Assay pH	K_i PALA (nM)
Hoogenraad (16)	mouse spleen	[U-^{14}C] Asp	Porter (3)	1.8	0.045	7.4	
				3.4	10	7.4	26
Baillon (17)	human lines:	[U-^{14}C] Asp	Porter (3)				
	fibroblasts			4	10	8.5	1.2
	melanoma			7	10	8.5	1.5
	colorectal			7.8	10	8.5	1.3
Moore (7)	human spleen	[U-^{14}C] Asp	Porter (3)	20.6	4	7.4	11
	rat liver			46.8	4	7.4	not done
Kensler (8)	human WBCs	[4-^{14}C] Asp	Jayaram (4)	9.5	5	8.5	4.5
Kensler (12)	murine Lewis lung cancer	[4-^{14}C] Asp	Jayaram (4)	0.5	1.25	8.4	20
Shoaf (15)	Ehrlich Ascites	[^{14}C] CP	Davies (5)/Bethell (6)	3–4	5	8.7	not done
Kempe (19)	SV40-transformed hamster line	[^{14}C] CP	Davies (5)/Bethell (6)	28	15	8.5	0.4
Present report	human peripheral blood mononuclear cells	[^{14}C] CP	HPLC	58	2	8.0	not done

Asp = aspartate; CP = carbamoyl phosphate.

penetrate cells, essentially precludes the option of measuring ACTase activity using intact cells.

We minimized the dilutional effects of cell preparation on PALA concentration, and the cytosol comprised 75% of the reaction mixture. Further, all patient samples were prepared in a uniform fashion and the amount of carbamoyl aspartate formed over time was normalized to the number of nucleated peripheral blood mononuclear cells. This method consistently detected enzyme activity in the cytosol of peripheral blood mononuclear cells isolated from 10 ml blood and we documented dose-dependent inhibition of ACTase activity 24 h after administration of PALA. ACTase activity could be quantitated, however, using cytosol from the equivalent of 300000 cells. Therefore, smaller blood volumes (2–5 ml) should suffice.

Conclusion

We developed a direct HPLC assay for the determination of ACTase activity in patients receiving PALA. This method proved more useful than a previously published assay because it eliminated the problems of high background counts, and ACTase activity was readily measurable in every patient sample. This method should also be suitable for measuring the biochemical effects of PALA in tumor tissue. Since this assay is more time consuming and requires more sophisticated equipment than previously published assays, it may not provide an advantage in situations in which excess carbamoyl phosphate can be employed, such as measurement of baseline ACTase activity in various PALA-sensitive or -resistant cell lines, or in tumor and adjacent normal tissue. When one is attempting to detect inhibition of ACTase activity in patient samples, however, this HPLC method does provide an advantage, since determination of the precise amount of carbamoyl phosphate is crucial. Since the product of the reaction, [^{14}C]carbamoyl aspartate, was stable once formed, the use of an autosampler with an abbreviated HPLC method (e.g. method 2) should increase its utility.

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