Application of a Simple Competitive Protein-binding Assay Technique to the Pharmacokinetics of N-(Phosphonacetyl)-l-aspartate in Humans

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ABSTRACT

A competitive protein-binding assay for N-(phosphonacetyl)-l-aspartate (PALA) using aspartate transcarbamylase as the receptor protein and [14C]PALA as the radioactive ligand is described here and has been applied to study the pharmacokinetics of PALA in humans. A protein-free ultrafiltrate of plasma, prepared by centrifugation of 1-ml samples through Amicon Centriflo membrane cones, was used in the assay, which had a maximum sensitivity of 0.7 μM PALA in plasma. At this level, the coefficient of variation was less than 10%. Comparison of the competitive binding assay to a gas chromatographic-mass spectrometric technique shows that the two methods yield equivalent results in the concentration range of 1 μM to 1 mM. However, the competitive binding assay possesses practical advantages because of its simplicity and the ease with which multiple samples may be assayed. PALA disappearance from plasma was studied in seven patients and was found to be consistent with a two-compartment open model. The t1/2α (elimination half-life for initial phase) and t1/2β (elimination half-life for terminal phase) were 0.93 ± 0.73 (S.D.) hr and 4.82 ± 1.48 hr, respectively. The cumulative urinary excretion of PALA in two patients was 70 and 90% of the administered dose 16 hr after the infusion was completed.

INTRODUCTION

PALA2 was synthesized by Collins and Stark (3) as a transition state inhibitor of ATC, the second enzyme in the de novo pathway of pyrimidine biosynthesis. Antitumor screening revealed that PALA had significant activity against Lewis lung carcinoma, B16 melanoma, and glioma 26 but was ineffective against L1210 leukemia (9). Studies of PALA disposition in animals indicated that the drug was not metabolized but was excreted intact in the urine and was found to localize in bone (2, 15). Phase I studies were carried out in humans and revealed that dose-limiting toxicity occurred in the skin and gastrointestinal tract (5).

PALA is a small, ionized, and relatively nonreactive molecule with little UV absorption. These characteristics make it difficult to develop a sensitive assay method which is sufficiently simple and rapid to be useful for routine clinical use. PALA assays using GC-MS (15) as well as inhibition of ATC (4, 6) have been described, but these methods have certain disadvantages. In the present report, we describe a rapid and simple CPBA for PALA which is based on the separation of bound and free PALA by adherence of the complex to nitrocellulose filters. This assay has been used in preliminary pharmacokinetic studies during Phase I and II trials of PALA. Furthermore, this method allows quantitation of the binding kinetics of PALA and its target enzyme.

MATERIALS AND METHODS

Materials. Unlabeled PALA was obtained from the Drug Development Branch of the National Cancer Institute. [14C]PALA (specific activity, 23 mCi/mmole) was obtained from the Stanford Research Institute, Menlo Park, Calif. The [14C]PALA was 97% pure by radioautography and 98% pure by 31P nuclear magnetic resonance. High-pressure liquid chromatography of the [14C]PALA yielded 95% pure compound. Imidazole, EDTA, and 2-mercaptoethanol were of standard reagent grade.

Amicon Centriflo membrane cones CF25 (Amicon Corporation, Lexington, Mass.) were used to prepare the samples for assay. Sartorius membrane filters (SM1 1310) composed of nitrocellulose acetate (2.5-cm diameter; pore size, 0.05 μm) and Whatman GF/C filters were purchased from Beckman Instruments, Inc., Science Essentials Operations, Mountainview, N. J.

Partially purified ATC from Escherichia coli (New England Enzyme Center, Boston, Mass.) was used throughout this study. The specific activity of this enzyme was 7.7 μmol of carbamylaspartate formed per mg of protein per hr at pH 8 and 37°, using the method of Porter et al. (11).

PALA samples containing 100 units of heparin per ml were obtained from patients receiving PALA i.v. in a 15-min infusion during Phase I and II trials (5). Urine was collected in 4- or 6-hr aliquots. All samples were stored at −15° until analysis was performed.

Method. The assay is based on competition between [14C]PALA and unlabeled PALA for binding to ATC. Protein-bound drug is separated from the unbound drug by filtration through the nitrocellulose acetate membrane filters, which selectively retain the enzyme-bound drug. The amount of bound labeled PALA can then be measured by scintillation counting of the filter and is inversely proportional to the amount of unlabeled PALA added. The drug concentration in the patient samples can then be calculated by comparison of bound counts to a standard curve which relates bound counts to known concentrations of PALA added to plasma.

Standards of known concentrations of PALA ranging from 0.5 to 50 μM were prepared in pooled plasma. Heparin was not added to the pooled plasma as it did not affect the standard curve. Aqueous standards were used in the case of the urine assays. The standards were stored at −15° until needed.

Plasma standards and test samples were prepared by cent-
filter cones at 1000 x g for 1 hr. This ultrafiltration step was necessary to remove the plasma proteins for which the nitrocellulose filters have a limiting capacity of 800 to 1000 μg/filter (16). Urine did not require preliminary ultrafiltration. Recovery of PALA in the ultrafiltrate was found to be 80 ± 1.4% (S.D.) in the concentration range from 0.1 μM to 1 mM.

The assay involved sequential addition of: (a) 300 μl of 80 mM imidazole acetate buffer, pH 7.0, with 4 mM 2-mercaptoethanol and 0.4 mM EDTA; (b) 100 μl of 2.2 μM [14C]PALA (specific activity, 23 mCi/mmol); (c) 100 μl of the protein-free filtrate of standard or serially diluted test sample; and (d) 100 μl of partially purified ATC with 0.28 nmol of [14C]PALA-binding capacity as determined under the assay conditions. This solution was incubated for 30 min at 37° to allow equilibrium to be achieved.

The nitrocellulose filters presoaked in the reaction buffer for at least 60 min were placed on top of the similarly presoaked Whatman GF/C filters sitting on the grids of the filter holder. A 500-μl aliquot of the reaction mixture was then placed onto the filters, and a controlled vacuum of —380 mm Hg was applied for 2 min. This filtration step was repeated after applying 500 μl of 40 mM imidazole acetate buffer, pH 7.0, with 2 mM 2-mercaptoethanol and 0.2 mM EDTA to the filter in order to remove excess unbound PALA. The nitrocellulose filters were then dissolved in 14 ml of Aquasol (New England Nuclear), and enzyme-bound [14C]PALA was determined in a liquid scintillation counter with a counting efficiency of 95%.

A standard curve was constructed with each series of patient samples, and the PALA concentration of unknown samples was determined by reference to these curves. Each sample was assayed in duplicate.

Calculations. The standard curves relating the concentrations of PALA in the plasma to the bound [14C]PALA may be constructed in 2 ways (Chart 1). A plot of the percentage of bound [14C]PALA versus log concentration of PALA in plasma results in a sigmoidal curve with a linear portion between 1.2 and 15 μM. By replotting the ordinate as the logit transformation (12), a linear standard curve from 0.74 to 36 μM PALA can be constructed. The logit transformation is defined as:

\[ \logit = \log \left( \frac{B/B_0}{1 - B/B_0} \right) \]

where \( B_0 \) is [14C]PALA bound in the absence of unlabeled PALA and \( B \) is [14C]PALA bound in the presence of a given amount of unlabeled PALA.

Pharmacokinetic data were fitted by nonlinear regression using the MLAB system implemented on the DEC-10 computer (10). The pharmacokinetic parameters were calculated by a programmable desk-top calculator using the equations outlined by Gibaldi and Perrier (7) which incorporate infusion time.

Analysis of the ATC-PALA equilibrium binding data was also carried out using the MLAB system. These data were then plotted according to the method of Scatchard (13).

RESULTS

Separation of Free from ATC-bound PALA. The nitrocellulose acetate membrane filters have the ability to retain protein and protein-bound ligands (16), as shown in Table 1. In the absence of ATC, the nonspecific retention of [14C]PALA by these filters was 1.7%. In the presence of ATC, bound [14C]PALA was 70% of the total amount of [14C]PALA added to each assay tube. In the presence of excess unlabeled PALA which will compete with the [14C]PALA for binding to ATC, the amount of [14C]PALA bound to the filters was reduced to 2% or approximately equal to the nonspecifically bound fraction in the absence of ATC. Thus, the nitrocellulose filters effectively separate protein-bound [14C]PALA from the unbound fraction.

Affinity of PALA for ATC. Binding of PALA to ATC was characterized in this system and was found to be optimal at pH 7.0. Under the standard assay conditions described in ‘Materials and Methods,’ competition for binding of [14C]PALA to ATC could be detected at minimum PALA concentrations of approximately 0.7 μM in plasma, and nearly complete displacement of the labeled drug from enzyme was achieved at 35 μM PALA in plasma (Chart 1). Binding equilibrium was reached by 30 min and was found to be stable for at least an additional 60 min.

Equilibrium binding studies were carried out in order to characterize the nature of PALA binding to ATC from E. coli. A plot of the bound/free ratio versus bound [14C]PALA yielded a convex upward curve (Chart 2). This finding suggests that PALA binding to ATC demonstrates positive cooperativity (14) and is in agreement with the characteristics of E. coli ATC reported by others (8).

CPBA of PALA. As shown in Chart 1, the lower limit of sensitivity of this assay was 0.7 μM in plasma. The coefficient of variation was less than 10% (n = 6) over the concentration range of 0.7 to 1000 μM. The specificity of the assay was tested by allowing [14C]PALA binding to occur in the presence of the natural substrates of the ATC-catalyzed reaction and in the presence of the reaction product. The substrates, carbamyl phosphate and aspartic acid, were added singly to assay solutions containing 0.22 nmol of [14C]PALA or were added together in equimolar concentrations from 0.1 μM to 1.0 mM. The product carbamylaspartate was added in concentrations from 0.5 μM to 0.5 mM. Chart 3 demonstrates that there was no significant interference with [14C]PALA binding to ATC ex-
Separation of protein-bound from free PALA

Each assay tube contained 0.28 nmol of \(^{14}C\)PALA (specific activity, 23 mCi/mmol), buffer, and where indicated Escherichia coli ATC with 0.3 nmol of PALA binding activity and 300 nmol of unlabeled PALA in a total volume of 600 µl. The solutions were incubated at 37° for 30 min and then filtered. Filter-bound counts were determined as described in "Materials and Methods."

<table>
<thead>
<tr>
<th>ATC</th>
<th>(^{14}C)PALA</th>
<th>PALA</th>
<th>(^{14}C)PALA bound to filter (dpm)</th>
<th>% bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
<td>248</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>287</td>
<td>2.0</td>
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Plasma disappearance of PALA was studied at dose levels of 600, 1000, 1500, and 2000 mg/sq m/day. The dose of 1500 mg/sq m/day was the recommended starting dose for patients treated daily for 5 days every 3 weeks (5). Chart 5 depicts the computer-fitted curves and the actual data points for plasma PALA concentrations in Patient 1 receiving 600 mg/sq m, Patient 2 receiving 1000 mg/sq m, and Patient 4 receiving 1500 mg/sq m. The plasma decay curves were best fitted by a biexponential function suggesting that a 2-compartment open model can adequately describe PALA disappearance over 24 hr. A summary of the pharmacokinetic parameters for the 7 patients is presented in Table 2. The mean t\(_{1/2}\beta\) (elimination half-life for terminal phase) was 4.82 ± 1.48 hr for

In order to assess the accuracy of this assay, 52 samples from patients treated with PALA in the Phase I study carried out at the National Cancer Institute were analyzed by the CPBA and by a previously described GC-MS method (15). Chart 4 compares the results of these 2 methods. An unweighted least-square linear regression yielded a close correlation (r = 0.89). More importantly, analysis by the Wilcoxon signed rank test (1) revealed that these results were not significantly different from each other (p ≤ 0.05) over the concentration range of 1 µM to 1 mM.

**PALA Pharmacokinetics.** PALA plasma pharmacokinetics were determined in 7 patients treated at the National Cancer Institute in Phase I and II trials of PALA. The patients received PALA i.v. in 15-min infusions daily for 5 days. PALA levels in plasma were monitored following the initial dose of the 5-day cycle. The creatinine clearances ranged from 48 to 92 ml/min in the 7 patients.

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the 7 patients studied. The C₀ (corrected for infusion time) ranged from 0.3 to 3.68 mM. PALA total body clearance ranged from 47.4 to 138 ml/min and was less than the creatinine clearance in Patient 2, but greater than the creatinine clearance in the other 5 patients. A least-square linear regression analysis of PALA clearance and creatinine clearance from Table 2 yielded a correlation coefficient of 0.6. The apparent volume of distribution at steady state was in the range of 0.218 to 0.305 liter/kg for 6 of the 7 patients, suggesting a distribution slightly in excess of the extracellular body compartment.

PALA urinary excretion was also examined in 2 patients. More than 90% of the cumulative PALA excretion occurred within 16 hr after completion of the infusion. The maximum amount of PALA dose recovered in the urine was 70% in Patient 7 treated with 2000 mg/sq m/day and 90% in Patient 3 receiving 1000 mg/sq m/day.

DISCUSSION

The CPBA for PALA reported in this paper has sensitivity comparable to those of the other methods recently described (4, 6, 15). We have compared it directly to the GC-MS technique and have found that our assay gives similar results. The GC-MS method is probably the most specific of the techniques available, but the initial costs of the apparatus and the technological complexities make the use of this method unsuitable for more routine multiple analysis. In the case of PALA derivation of the parent compound with an extensive clean-up procedure makes it impossible to assay samples in one working day. On the other hand, the CPBA as described allows the analysis of 25 to 30 patient samples in duplicate during one working day. The enzymatic technique described by Cooney et al. (4) for measuring PALA in tissues can be adapted to plasma and urine. The sensitivity of this method appears to be better than the CPBA, but the authors caution against possible interference by substances such as L-aspartic acid. Furthermore, the method requires a 3-hr decarboxylation step to allow the release of 14CO₂ from unreacted aspartic acid. Friedman et al. (4) have reported a similar enzyme technique for PALA. The lower limit of detectability in plasma was 0.38 μM for their method, but the coefficient of variation was between 9 and 17% at this level. These authors also suggest a potential for interference in this method by unidentified substances. Although it is conceivable that unknown material may interfere with the CPBA, we could not demonstrate any significant interference by the natural substrates of the reaction which are most likely to be the unidentified source. Thus, the CPBA combines simplicity and rapidity with specificity, sensitivity, and precision in the measurement of PALA in plasma and can be readily utilized for routine drug monitoring.

We have been able to fit a 2-compartment model to the plasma disappearance of PALA and to determine various pharmacokinetic parameters (Table 2). Our data suggest that there may be a correlation between PALA clearance and creatinine clearance as would be expected with a drug which is cleared in unchanged form by the kidney. However, this correlation will need further corroboration by the analysis of more patient material. In light of this suggestion, some dose modification is probably in order for patients with compromised renal function.

The mean volume of distribution of 0.333 ± 0.185 liter/kg is slightly larger than extracellular fluid volume in humans, suggesting that PALA has limited access to many tissue compartments. Such a finding may explain the need for high doses of PALA in order to produce biological effects. Prolonged PALA infusions or repeated doses at frequent intervals might maintain

Table 2

<table>
<thead>
<tr>
<th>Patient</th>
<th>PALA dose (mg/sq m/day)</th>
<th>Creatinine clearance (ml/min)</th>
<th>PALA clearance (ml/min)</th>
<th>α (min⁻¹)</th>
<th>β (min⁻¹)</th>
<th>t₁/₂β (hr)</th>
<th>Vₘ (liter/kg)</th>
<th>C × t (ms⁻¹)</th>
<th>C₀ (mm)</th>
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<td>600</td>
<td>66</td>
<td>86.7</td>
<td>0.020</td>
<td>0.0031</td>
<td>3.72</td>
<td>0.305</td>
<td>33.4</td>
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</tr>
<tr>
<td>2</td>
<td>1000</td>
<td>48</td>
<td>44.4</td>
<td>0.006</td>
<td>0.0022</td>
<td>5.25</td>
<td>0.303</td>
<td>113.0</td>
<td>0.587</td>
</tr>
<tr>
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<td>1000</td>
<td>64</td>
<td>118</td>
<td>0.016</td>
<td>0.0022</td>
<td>5.25</td>
<td>0.744</td>
<td>38.1</td>
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<tr>
<td>4</td>
<td>1500</td>
<td>92</td>
<td>138</td>
<td>0.073</td>
<td>0.0043</td>
<td>2.69</td>
<td>0.241</td>
<td>71.6</td>
<td>2.05</td>
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<tr>
<td>5</td>
<td>1500</td>
<td>63</td>
<td>90.3</td>
<td>0.008</td>
<td>0.0022</td>
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<td>0.302</td>
<td>85.9</td>
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<td>75</td>
<td>71.4</td>
<td>0.005</td>
<td>0.0016</td>
<td>7.22</td>
<td>0.221</td>
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<td>0.649</td>
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<tr>
<td>7</td>
<td>2000</td>
<td>57</td>
<td>47.4</td>
<td>0.069</td>
<td>0.0028</td>
<td>4.08</td>
<td>0.218</td>
<td>247.0</td>
<td>3.68</td>
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</table>

α: PALA clearance, total-body PALA clearance; β: disposition rate constant for initial phase; t₁/₂β: elimination half-life for terminal phase; Vₘ: apparent volume of distribution at steady state; C × t, area under the curve fitted to a 2-compartment model.
high plasma levels and might be able to increase tissue uptake and improve therapeutic results.

Although we could not conclude that there was a relationship between pharmacokinetic parameters and toxicity or therapeutic effect, there was a suggestion that the C₀ may be directly correlated with toxicity (Table 3). Patients 4 and 7 who developed severe skin rashes, profuse diarrhea, and stomatitis had the highest C₀ values of 2.05 and 3.68 mM, respectively. Patients 2, 5, and 6, who experienced only moderate skin rash, had C₀ values between 0.5 and 0.7 mM. No toxicity was observed in Patients 1 and 3, who had C₀ values of less than 0.5 mM. Furthermore, Patient 3, who had significant hepatic impairment, did not have a prolongation of the t₁/₂, a finding consistent with the fact that there is little if any hepatic metabolism of PALA. This suggests that dose modification is not necessary in patients treated with PALA who have impairment of hepatic function.

The method that we describe can be adapted to study the characteristics of PALA binding to ATC through equilibrium analysis. This approach may give more detailed information regarding the binding of PALA to ATC when compared to the results of classical enzyme kinetic studies, since it allows direct measurement of the enzyme-drug complex.

This technique has potential applicability in the clinical setting. Since all evidence available suggests that PALA is eliminated through the urine, monitoring patients with compromised renal function may allow optimization of PALA administration in terms of toxicity. On the schedule used in the present studies (i.e., treatment daily for 5 days), the rapid determination of PALA plasma levels during the first day of drug administration would identify patients with impaired drug excretion and may thus allow adjustment of the subsequent doses in a cycle. Finally, PALA as an inhibitor of de novo pyrimidine biosynthesis has potential for synergism with 5-fluorouracil and other pyrimidine analogs. Although this synergism is based on the biochemical actions of these drugs, the potential for drug interaction resulting in altered pharmacokinetics cannot be ruled out. Therefore, a simple method of assessing any changes in PALA pharmacokinetics will be of value in these studies.

ACKNOWLEDGMENTS

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REFERENCES


Table 3

<table>
<thead>
<tr>
<th>Patient</th>
<th>PALA dose (mg/sq m)</th>
<th>C₀ (mM)</th>
<th>Toxicity</th>
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<tr>
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<td>0.587</td>
<td>Moderate</td>
</tr>
<tr>
<td>3</td>
<td>1000</td>
<td>0.329</td>
<td>None</td>
</tr>
<tr>
<td>4</td>
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<td>Severe</td>
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<tr>
<td>5</td>
<td>1500</td>
<td>0.621</td>
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</tr>
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<tr>
<td>7</td>
<td>2000</td>
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