

# Determination of *N*-(Phosphonacetyl)-L-Aspartic Acid (PALA) in Plasma and Urine by High Pressure Liquid Chromatography\*

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**Abstract**—A rapid and sensitive determination of *N*-(phosphonacetyl)-L-aspartic acid (PALA) in plasma and urine using high pressure liquid chromatography is reported. A microparticulate, strong anion exchange column was used for the separation and PALA was detected by u.v. absorption at 205 nm. Interfering substances were removed from deproteinized plasma or from urine by a simple and quick clean-up procedure involving extraction with organic solvents. The detection limit for PALA was  $5 \times 10^{-7}$  M and linear dynamic range was greater than  $10^4$ . The assay time required was less than an hour. Plasma concentrations and urinary excretion of PALA were measured using this method in two patients treated with PALA. The ease and quickness of this assay are advantages over reported methods and make possible close monitoring of the pharmacokinetics of this drug.

## INTRODUCTION

*N*-(phosphonacetyl)-L-aspartic acid (PALA) is a transition state inhibitor of aspartate transcarbamylase and blocks *de novo* pyrimidine biosynthesis [1]. PALA has been demonstrated to be active against several murine tumor models [1-3] and is currently being investigated in man. The study of its pharmacokinetics may elucidate correlations with its toxicity, as has been shown for methotrexate [4], or with its anti-tumor effect. Assays for the measurement of PALA in biological fluids using enzymatic techniques [5, 6] or gas chromatography/mass spectrometry (GC/MS, [7]) have been described offering high specificity and sensitivity. However, the enzymatic assays have the disadvantage of a narrow linear range, while the gas chromatography method requires prior chemical derivatization of the sample to convert PALA to a volatile form. This requires extra time and a high number of handlings. In the method described here, the number of handlings during pretreatment of the sample

is reduced and results in a low standard deviation for the height of the PALA peak in the chromatogram. Therefore the use of an internal standard is not required in our method, in which high pressure liquid chromatography (HPLC) is used as an analytical tool. The detection limits lie in the same order of magnitude for both GC/MS and HPLC methods. When comparing the equipment of both chromatographic methods, HPLC equipment is less expensive. A sample assayed with the method described here can be performed within 40 min.

## MATERIALS AND METHODS

### Chemicals

PALA was obtained from the National Cancer Institute, Bethesda, Md., U.S.A. Acetonitrile (Uvasol quality) obtained from Merck (Darmstadt, W. Germany) and de-ionized, glass-distilled water were used in the preparation of the eluent. All other chemicals were of analytical grade.

### HPLC apparatus

The chromatographic system consisted of a high pressure pump (Orlita, Type DMP AE 10.4), a high pressure injection valve (Valco,

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Type CV-6-UHPa-N60) and a variable wave length detector (Perkin-Elmer, Type LC75). The use of a flow-through pressure gauge provided sufficient damping of the pressure pulse of the pump. A microparticulate, strong anion exchange column (Partisil, SAX, Whatman) with a  $10\text{ }\mu\text{m}$  particle size was used. The column dimensions were: length 25 cm and i.d. 4.6 mm. This column was replaced every 2 months due to loss of performance. A pre-column filled with activated charcoal was used to remove organic contaminants from the eluent. The temperature of the analytical column was controlled by a column thermostat block (Knauer, Type 75,00).

#### Sample clean-up

Heparinized blood was centrifuged at room temperature at 2000 *g* for 5 min. Plasma or urine (0.8 ml) was deproteinized by adding 0.2 ml 2N perchloric acid while mixing with a vortex mixer. The acid-precipitated material was removed by centrifugation. The acid-soluble fraction was subjected to three extraction steps to remove substances that interfered with the detection of PALA after the chromatographic run. The first extraction involved mixing 0.7 ml of sample with 0.2 ml solution of 2 mg/ml sodium dodecylhydrogen sulfate (SDS) and 1 ml chloroform for 15 sec. The lower chloroform layer was removed and the aqueous phase was extracted with 1 ml fresh chloroform. For the second extraction step the aqueous phase, 0.6 ml, was mixed with 1 ml of a tributylphosphate-hexane mixture (1/1, v/v) for 15 sec. The upper organic phase was removed and 0.5 ml of the aqueous phase was extracted in the third step with 0.5 ml tributylphosphate for 15 sec. The organic phase was removed and an aliquot of the lower aqueous phase was injected into the high pressure liquid chromatograph. All the above extractions were performed using a vortex mixer and phases were separated by centrifugation at 2000 *g* for 0.5 min.

## RESULTS AND DISCUSSION

The first problem encountered in measuring PALA with HPLC was the lack of a good selective u.v. absorption wavelength. Figure 1 shows that PALA does not appreciably absorb at wavelengths above 230 nm. Detection at 205 nm was selected, despite higher absorption at lower wavelengths, because of better baseline stability.

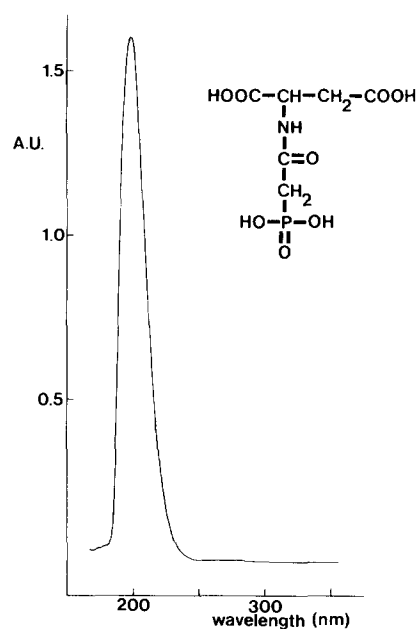


Fig. 1. Structure and u.v. spectrum of PALA; concentration  $3 \times 10^{-4}\text{ M}$  in 0.01 M  $\text{K}_2\text{H}/\text{KH}_2$  phosphate buffer, pH 7.

#### Chromatographic and detection conditions

PALA was eluted with a mixture of 9 parts 0.2 M potassium phosphate, pH 4.6, and 1 part acetonitrile (w/w) at a column temperature of  $40^\circ\text{C}$ , a flow rate of 2.7 ml/min and at a pressure of 140 bar. The capacity factor for PALA was 5.5. Figure 2 illustrates a representative HPLC separation of a plasma sample containing PALA. Using the above conditions PALA was retained just long enough to be separated from the large unretained peaks without being too diluted, resulting in optimal resolution and sensitivity. Retention time could be increased by lowering the phosphate concentration or increasing the pH of the eluent. The addition of acetonitrile to the eluent was necessary to reduce peak tailing by decreasing absorption effects. The elevated column temperature served to control peak broadening and decrease eluent viscosity. Up to  $350\text{ }\mu\text{l}$  of treated sample could be injected for analysis. Volumes exceeding  $350\text{ }\mu\text{l}$  resulted in peak broadening and interference from the negative peak caused by perchlorate occurring after the PALA peak. Peak height was used for quantitation of PALA. For a plasma concentration of  $10^{-3}\text{ M}$  the S.D. in peak height was 2.1% ( $n=5$ ) and for  $10^{-6}\text{ M}$  4.4% ( $n=5$ ). The detection limit was  $5 \times 10^{-7}\text{ M}$  for an injection volume of  $350\text{ }\mu\text{l}$ . Urine samples with high PALA concentrations of  $>10^{-4}\text{ M}$  were injected directly without any pretreatment. The standard curve was linear to at

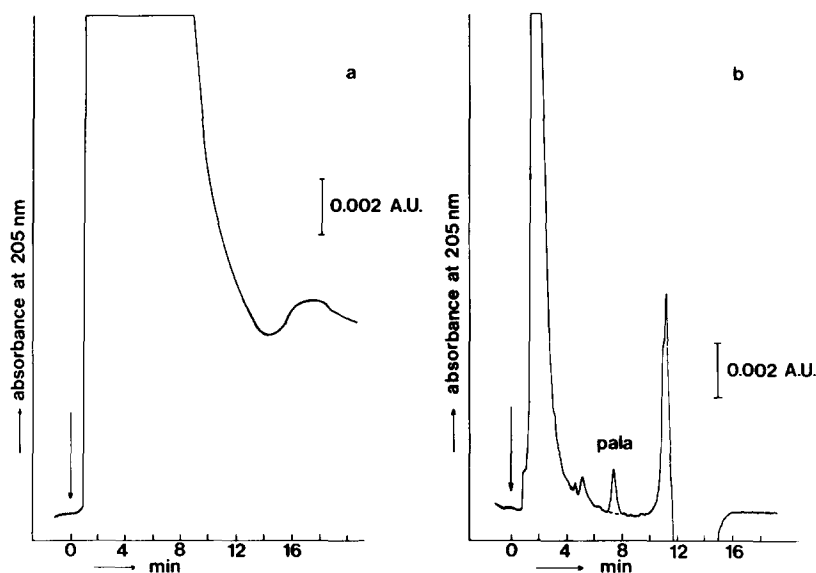


Fig. 2. (a) Chromatogram after injection of 350  $\mu$ l deproteinized plasma. (b) Chromatogram after injection of 350  $\mu$ l deproteinized plasma, extracted by three sequential washing steps (see Table 1); conditions: PALA concentration  $1.6 \times 10^{-6}$  M, eluent: potassium phosphate (0.2 M, pH 4.6) + acetonitrile (9+1, w/w), column: Partisil SAX, length 25 cm, i.d. 4.6 mm, particle diameter 10  $\mu$ m, eluent flow rate 2.7 ml/min, pressure 140 bar; broken line: plasma without PALA.

least  $5 \times 10^{-3}$  M indicating a linear dynamic range of over  $10^4$ .

Attempts to use other HPLC modes failed. PALA could not be retained on a reverse phase column under various conditions differing in pH and concentration of organic modifier, apparently due to its highly ionized nature (see Fig. 1). Although with soap chromatography [8] retention time could be controlled, this system proved to be unsuitable because of the interfering absorption of the agents for ion-pair formation (such as cetrimide) at the low wavelength used for detection.

#### Sample pretreatment

Samples were extracted with organic solvents to reduce the presence of interfering compounds. The first extraction was performed on perchloric acid treated samples (pH  $\sim 1$ ) with sodium dodecylhydrogen sulfate and chloroform to remove cations by ionpair extraction. The second and third extractions with organic solvents of increasing polarity removed non-polar substances. During the sample pretreatment procedure the PALA concentration decreases by the addition of perchloric acid and SDS solutions and possible minor transfer of PALA to the different organic phases. On the other hand the con-

centration may increase by the decrease in volume of the aqueous layer by solution of water in the organic phase or removal of proteins (PCA step). The change in PALA concentration during the pretreatment of a plasma sample is given in Table 1. The ultimate concentration in the aqueous layer is 79% of the initial concentration. Systematic variation in peak height caused by variables during pretreatment and chromatographic steps is compensated by measuring standard plasma samples ( $10^{-4}$  M PALA, at the beginning and the end of a run of samples per day). The clean-up procedure required less than 30 min and was sufficient to make PALA detectable. The use of citrate as anticoagulant should be avoided for blood samples, since citrate absorbs at 205 nm and interferes with the detection of PALA.

#### Interference by other chemotherapeutic agents

For clinical use PALA is likely to be combined with other chemotherapeutic agents acting upon pyrimidine synthesis, such as 5-fluorouracil, methotrexate and cytosine arabinoside. These compounds might interfere in the chromatogram. Under the presented chromatographic conditions the retention times of these compounds were measured and appeared to be much shorter relative to PALA.

Table 1. Changes in PALA concentration during sample pretreatment\*

	Fractional PALA concentration from initial concentration
After addition of perchloric acid	0.93
After addition of SDS solution:	
1st extraction with chloroform	0.73
2nd extraction with chloroform	0.72
After extraction with hexane/tributylphosphate	0.77
After extraction with tributylphosphate	0.79

\*For conditions as mentioned under *Sample clean-up*.

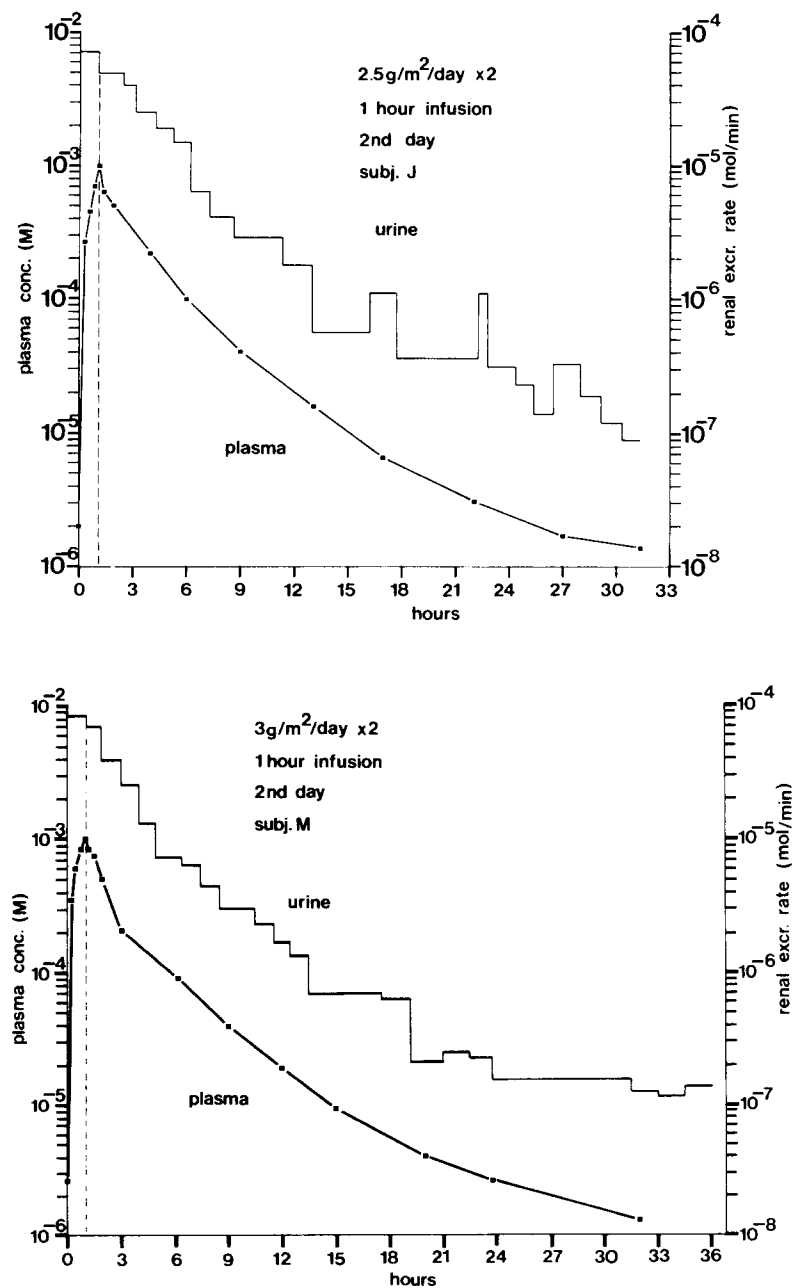


Fig. 3. Plasma concentration and renal excretion rate of PALA in two patients after a 1 hr infusion of PALA, given 2 sequential days; urine had been collected at the end of each horizontal level.

*Clinical application*

In Fig. 3 the PALA plasma concentration vs time curves are presented for two patients who received a PALA infusion of 1 hr in a phase 2 clinical trial in melanoma patients. The data were determined manually according to a three compartment fit [9]. Half-life times of 1 hr, 3.9 hr, 11.7 hr and 0.2 hr, 3 hr, 10.8 hr were found for patients M and J, respectively. After 24 hr infusions a biphasic plasma disappearance has been reported with half-life times ranging from 0.7 to 8.3 hr for the first phase and 5 to 13 hr for the last phase [10]. From the data of the patients studied here, it appears especially important to continue taking samples for more than 30 hr in order to get more information on the third phase.

Urinary excretion parallels the plasma con-

centration curve over the whole measured range (Fig. 3). From the renal excretion vs plasma concentration plot the renal clearance was found to be 117 and 96 ml/min for patients M and J, respectively. Total renal excretion amounted to 106 and 85% of the administered dose of PALA, indicating minimal metabolism, which is in agreement with earlier findings [3, 10]. Of the total excreted amount 95% was excreted in 10 hr. From patient M saliva and sweat was collected. The concentration was below  $5 \times 10^{-7}$  M.

If correlations between pharmacokinetics and toxicity or therapeutic efficacy are established a quick assay may be helpful, especially during PALA treatment.

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