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MASS SPECTROMETRIC TECHNIQUE FOR THE DETERMINATION OF N-PHOSPHONOACETYL-L-ASPARTIC ACID IN SERUM

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SUMMARY

N-Phosphonoacetyl-L-aspartic acid (PALA), a potent inhibitor of aspartic acid transcarbamylase, is now undergoing Phase I clinical trials. Initial experiments revealed that PALA is not metabolized to phosphonoacetic acid (PAA) in humans. Thus PALA may be quantified in serum after in vitro conversion to PAA. Serum is deproteinized with perchloric acid, lipid extracted with methylene chloride, hydrolyzed with 8 *N* hydrochloric acid at 100° for 3 h, and evaporated to dryness with nitrogen. The residue is silylated, and PAA is quantified by monitoring the $(M+1)^{+\cdot}$ ions of the protonated molecular ions of trimethylsilyl derivatives of PAA and phosphonopropionic acid (internal standard) obtained in chemical ionization with methane. Limit of detection is 0.5 μM (150 ng/ml) PALA using 1 ml serum. PALA was given by continuous infusion to cancer patients at various doses. Maximum levels of PALA (50—500 μM range) were obtained at the end of infusion, followed in most cases by biexponential decay. Persistent residual PALA levels (5 μM for 48 h after infusion) correlated with increased toxicity.

INTRODUCTION

Drugs that interfere with the de novo biosynthesis of pyrimidine nucleotides have proved useful in the treatment of a number of human tumor types. For example, orotidylate decarboxylase is a target of azapyrimidine action, thymidilate synthetase is the target of fluoropyrimidine action, and DNA polymerase is inhibited by cytosine arabinoside. The first committed reaction in the pathway of pyrimidine biosynthesis is the irreversible carbamylation of L-aspartate by carbamylphosphate to form carbamylaspartate (Fig. 1). This reaction is catalyzed by the enzyme aspartate transcarbamylase (ATCase). In 1971, Collins and Stark [1] synthesized N-phosphonoacetyl-L-aspartic acid (PALA, NSC 224131) which acts as a transition-state inhibitor of ATCase, either competitively with carbamylphosphate or noncompetitively with L-aspartate [2].

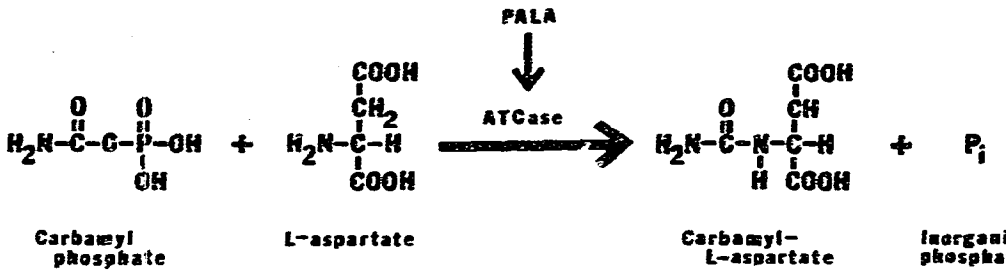

Fig. 1. N-Phosphonoacetyl-L-aspartic acid (PALA) is an inhibitor of aspartate transcarbamylase (ATCase) catalyzed formation of carbamyl-L-aspartate from carbamylphosphate and L-aspartate.

PALA was next shown to be cytotoxic to cultured mammalian cells, and reversal experiments with exogenous uridine or carbamyl-DL-aspartate proved that the cytotoxicity was indeed caused by interference with ATCase [3, 4]. The spectrum of antitumor activity of PALA in mice appears quite different from that of other antimetabolites: there is strong chemotherapeutic activity against several slow growing rodent tumors in contrast to inactivity to the fast-growing experimental leukemias [5].

With the beginning of Phase I clinical trials with PALA at several institutions, there have been concurrent attempts to develop techniques for quantification. In one technique [6] PALA is detached from ATCase by heating, proteins are removed, and PALA is quantified in terms of newly formed [^{14}C]-carbamyl-L-aspartic acid after incubation with intact splenic ATCase, L-[4-^{14}C]aspartic acid, and carbamylphosphate and enzymatic removal of unreacted aspartic acid. From the percentage inhibition of ATCase PALA may be assayed down to 0.1 μM concentration. In another enzymatic assay [7] the inhibition of partially purified aspartate carbamyltransferase from rat liver is utilized; detection limit is 0.1 μg/ml (0.4 μM). These techniques are relatively simple and quite sensitive and certainly adequate in many applications. They do suffer from problems involving enzyme purifications, certain interferences, and a need to run several replicates to obtain averages for a linear calibration curve; similarly, several runs must be made on each patient sample for adequate precision.

The tetramethyl ester of PALA can be formed with diazomethane but the electron impact mass spectrum of the compound exhibits only a weak molecular ion. Based on initial work on the chromatographic and mass spectrometric properties of permethylated PALA [8] a technique was described for quantification utilizing certain fragment peaks and ^{13}C-labeled PALA as the internal standard [9]. The limit of detection of this technique is 2 μg/ml (5.6 μM) which is inadequate to quantify PALA during the decay phase after terminating drug infusion. In addition, monitoring fragment peaks only at the relatively low mass of m/e 220, where endogenous interferences often occur, does not provide specificity.

Our initial experimentation with permethylation gave results similar to those described above. Direct trimethylsilylation of PALA appeared promising at the beginning when chemical ionization was employed to obtain the protonated molecular ion; however, when selected ion monitoring was attempted to in-

crease sensitivity, interferences occurred. Initial experiments with high-performance liquid chromatography revealed difficulties in providing a specific and sensitive technique. In connection with a preclinical toxicological study on phosphonoacetic acid (PAA) we have developed a technique for the quantification of PAA based on selected ion monitoring [10]. After establishing that PAA is not an in vivo metabolite of PALA (see Results and discussion), we have developed a technique for the quantification of PALA in human serum in terms of PAA which is a product of the in vitro hydrolysis of PALA. This paper describes the details of the methodology and illustrates applications.

EXPERIMENTAL

Drugs and reagents

Pure PALA (NSC 224131) as both the disodium and tetrasodium salt, was provided by the National Cancer Institute, Bethesda, Md., U.S.A. Most work was carried out with the disodium salt; the tetrasodium salt gave identical results. For clinical work PALA was also supplied by the National Cancer Institute. Ampoules containing 1000 mg PALA in 10 ml normal saline (100 mg/ml PALA) were used. The pH was adjusted to 6.5—7.5 with sodium hydroxide. Intact ampoules were kept refrigerated at 2—8° and unused portions were discarded. Pure phosphonoacetic acid was purchased from Richmond Organic (Richmond, Va., U.S.A.); pure phosphonopropionic acid was purchased from K & K Rare and Fine Chemicals (ICN Pharmaceuticals, Plainview, N.Y., U.S.A.). N,O-Bis(trimethylsilyl)trifluoroacetamide and trimethylchlorosilane (BSTFA + 1% TMCS), silylation grade pyridine and all gas chromatographic column materials were purchased from Pierce (Rockford, Ill., U.S.A.). All solvents used were of "distilled in glass" quality (Burdick & Jackson Labs., Muskegon, Mich., U.S.A.). All other chemicals were of highest purity commercially available and were used without further purification. Gas chromatographic carrier gases and reagent gases for chemical ionization mass spectrometry were of high purity grade from Matheson (Rutherford, N.J., U.S.A.).

Instrumentation

The instrument used was a combined gas chromatograph—mass spectrometer (quadrupole-type mass analyzer, Finnigan Model 3300)—computer (Finnigan Model 6000) system equipped with a chemical ionization source and capability for selected ion monitoring (mass fragmentography).

Internal standard

Phosphonopropionic acid (PPA) was used as the internal standard. The pure compound was dissolved in distilled water to provide solutions containing either 500 ng/μl or 50 ng/μl PPA. In all cases, an adequate amount of internal standard was added at the beginning of an analysis to yield a final concentration approximately 75% of the expected concentration range of PALA.

Standards and calibration samples

Pure PAA and PPA samples were dissolved in a mixture of BSTFA+1% TMCS and pyridine (3:1, v/v) and derivatized as described in Preparation of

serum samples. These samples were used to establish authentic mass spectra under various experimental conditions, to determine detection limits for the pure compounds, to establish sensitivities (in terms of computer-generated peak areas per unit sample quantity introduced) and also for daily routine mass range calibration.

To establish optimal analytical conditions both for sample preparation and also for gas chromatographic—mass spectrometric analysis, normal pooled serum and also normal individual serum samples were spiked with known quantities of PALA and PPA. To obtain calibration curves, samples were prepared containing increasing quantities of PALA and a fixed amount of internal standard. The concentration range of the calibration samples covered the entire range of concentrations expected in the patient samples. The amount of internal standard added was the same for both calibration and patient samples. Blank samples (i.e., no PALA added but PPA included) were also included in every set of calibration runs. A full set of calibration samples was analyzed with every set of samples from patients to compensate for irreproducible experimental errors.

Preparation of serum samples

Blood samples for PALA were drawn from the contralateral arm at various intervals according to the clinical protocol followed in this investigation.

Serum was obtained by letting whole blood clot at room temperature for 20—25 min followed by centrifugation at approx. 500 *g* at room temperature for 10 min. Serum samples were stored at −80° until used. Samples from mice were obtained the same way, pooling the serum of 2—5 animals treated with the same dose of PALA.

To a 0.5-ml serum sample the internal standard was added, followed by vortexing (fast) for 15 sec. Next, 25 μl of perchloric acid (70%) was added and again vortexed (fast) for 15 sec. The fine protein precipitate became tightly packed after centrifuging at 30,000 *g* at 10° for 15 min. The clear supernatant (approx. 0.4 ml) was transferred into a small tube, acidified by adding 0.5 ml concentrated hydrochloric acid, and lipid extracted by adding 1.0 ml methylene chloride and vortexing. For best separation of the aqueous and organic layers, the mixture was centrifuged at 2000 *g* at room temperature for 5 min. (A small layer of emulsion at the interface is acceptable.) The clear upper layer was next pipetted into a 6-ml PTFE-capped silylation vial and heated in an aluminum heating block at 100° for 3 h. At the end of the hydrolysis, the sample became amber colored with occasional black specs which were ignored. Next, the sample was evaporated to dryness with dry nitrogen in a water bath at 50—60°. The remaining residue was a somewhat crusty brown solid. The evaporated samples were kept overnight in a vacuum desiccator filled with solid potassium hydroxide and Drierite. Prior to mass spectrometric analysis, the samples were silylated by adding 200 μl of BSTFA + 1% TMCS—pyridine (3:1, v/v) and refluxing in a dry heating block kept at 100° for 5—8 min. After derivatization some solids did remain at the bottom of the silylation vial; the liquid phase was brown but clear.

When the lowest limit of detection was attempted, a 1.0-ml starting sample size was used and the following changes were made in the procedure described.

Proteins were precipitated with 50 μl perchloric acid, hydrolysis was done with 0.8 ml concentrated hydrochloric acid, lipids were extracted with 3 ml methylene chloride and 600 μl derivatization reagent were added. After silylation the supernatant was decanted into a clean vial, the excess reagent was evaporated with nitrogen, and the residue was reconstituted with 100 μl of reagent; an aliquot of this was introduced into the gas chromatograph. It is noted that the final samples appeared darker in this procedure than in the one commonly used; this did not alter results.

Patient samples

Blood samples were obtained from cancer patients participating in a Phase I clinical study. Patient selection, the mechanism of the study, dose escalations, consent forms, methods of evaluation, etc. were according to a clinical protocol approved by the Institution and submitted to the National Cancer Institute. The primary objective of the study was to establish the maximum tolerated dose of PALA in patients with cancers not amenable to conventional treatment methods. In a coordinated effort with other institutions, emphasis was placed upon the study of the effects of 24-h and 5-day continuous infusion. In the former case dose levels were escalated from 0.5 to 10.5 g/m^2/24 h; in the latter case dose levels were escalated from 4 to 8.7 g/m^2/5 days.

Gas chromatography—mass spectrometry

For gas chromatographic separation a glass column (1 m × 2 mm I.D.) filled with 3% OV-17 on Chromosorb W HP (80—100 mesh) was employed. The column was operated isothermally at 150°; injector temperature was kept at 250°. Endogenous constituents trapped by the column at the low operating temperature were removed by periodically heating the column to 250° and keeping it at that temperature until no more eluent could be detected. The sample port was cleaned after every 20—25 analyses by removing 1—2 cm of column material (dark deposits) from the top of the column.

Methane gas was used both as the gas chromatographic carrier gas and as the reagent gas in the chemical ionization source of the mass spectrometer. There was no separator between the gas chromatograph and the ion source; the connecting tube was kept at 240°. The pressure of methane in the chemical ionization source was kept at approximately 1.3 mbar (uncorrected). Operational parameters of the mass spectrometer were adjusted daily for maximum sensitivity (at a resolution of about 400) using pure PAA samples. When extreme adjustments of the ion source became necessary to maintain desired sensitivity, the ion source was cleaned.

Full chemical ionization mass spectra for identification were obtained by operating the instrument in the "full scanning" mode. The "selected ion monitoring" mode was used for quantification in patient samples. Normally, 4-μl sample aliquots were injected into the gas chromatograph. The effluent was vented for a period of 30 sec to avoid contamination of the ion source by the excess silylation reagent and pyridine. Next, the effluent entered the ion source and ions at *m/e* 371 and *m/e* 357 were monitored; for additional confirmation of identity, peaks at *m/e* 355 and *m/e* 341 were also monitored occasionally (see Results and discussion).

RESULTS AND DISCUSSION

Hydrolysis of PALA

The basis of the present technique is the in vitro hydrolysis of PALA in serum to yield PAA. Because it is conceivable that PALA might hydrolyze to PAA in vivo, i.e., that PAA might be a metabolite of PALA, a search was made to detect PAA in the serum of patients receiving PALA at various doses. No PAA was detected in the blood of any patient. The limit of detection of PAA in serum is 20 ng/ml using a 0.2-ml sample size [10]. A series of experiments was also carried out with mice injected with various doses of PALA; no PAA was detected. It is concluded that PAA is not an in vivo metabolite of PALA. Other workers also failed to detect metabolites of PALA using ^{14}C-labeled drug [11, 12].

When heated with 8 *N* hydrochloric acid at 100° PALA hydrolyzes to yield PAA and aspartic acid (Fig. 2). This was confirmed by obtaining the trimethylsilyl (TMS) derivatives of the hydrolysis products. The protonated molecular ion of PAA appeared at *m/e* 357 (see later). The protonated molecular ion of aspartic acid appeared at *m/e* 350 (Fig. 3); as expected, three TMS groups were taken up by the molecule.

The aspartic acid obtained in the hydrolysis of PALA is not suitable for monitoring since there is a considerable amount of endogenous aspartic acid present in human blood. Fig. 4A illustrates the monitoring of the protonated

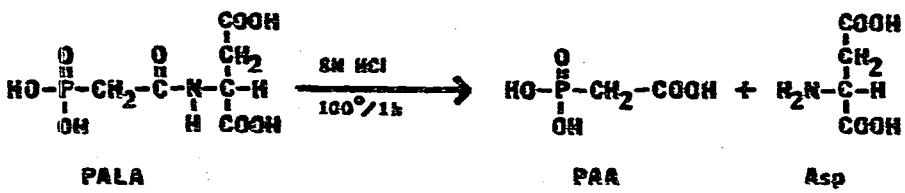

Fig. 2. In vitro hydrolysis of N-phosphonoacetyl-L-aspartic acid (PALA) to phosphonoacetic acid (PAA) and aspartic acid (Asp).

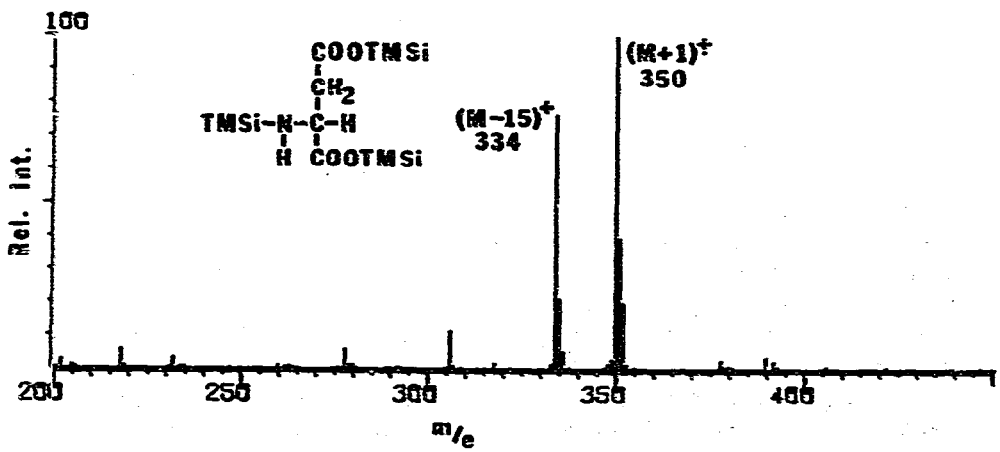

Fig. 3. Chemical ionization (methane) mass spectrum of the trimethylsilyl derivative of aspartic acid.

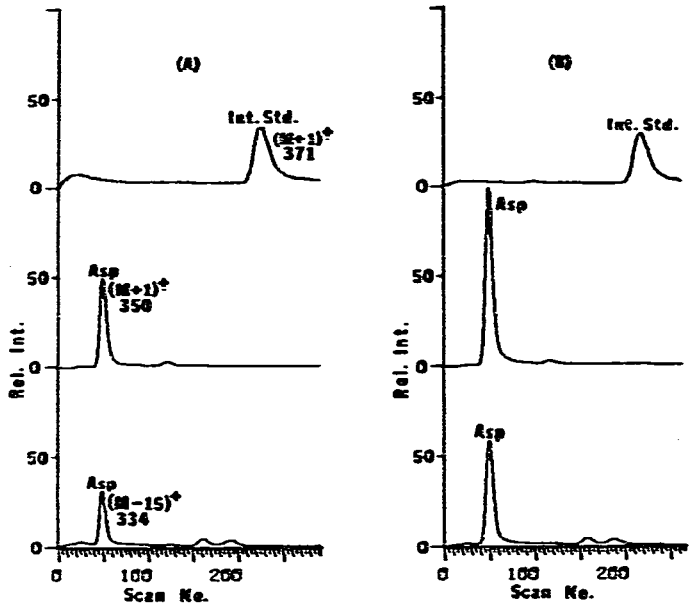

Fig. 4. Selected ion monitoring of the trimethylsilyl derivatives of aspartic acid (Asp) and internal standard (phosphonopropionic acid) in normal serum (A) and in serum spiked with PALA (B).

molecular ion of aspartic acid in normal blood. The $(M-15)^+$ ion, corresponding to the loss of a methyl group, was also monitored for confirmation. The area under the $(M+1)^{+\cdot}$ peak corresponds to approximately 50 μg/ml endogenous aspartic acid. When the same sample was spiked with 100 μg/ml PALA and hydrolyzed, the level of aspartic acid increased (Fig. 4B). Endogenous aspartic acid is a limiting factor in this approach for two reasons: endogenous aspartic acid is variable from patient to patient, and the limit of detection would be poor because of high blank values. In contrast, the zero-time or blank level of the peak at *m/e* 357 was found either below detection limit or at the few ng/ml level in all patient and normal samples analyzed. It was thus concluded that monitoring PAA in hydrolyzed serum samples can be utilized to quantify PALA.

Mass spectra of PAA and PPA

The chemical ionization (methane) mass spectra of pure silylated PAA (Fig. 5) and PPA (Fig. 6) reveal that the base peak in both cases corresponds to the protonated molecular ions with three trimethylsilyl groups taken up. The $(M+1)^{+\cdot}$ ions, at *m/e* 357 for PAA and *m/e* 371 for PPA, are thus well suited for selected ion monitoring. This technique increases sensitivity while retaining a high degree of specificity. As shown in the figures, the $(M-15)^+$ ions, corresponding to the loss of a methyl group, are also present in considerable abundance. These peaks may also be monitored, and the ratio of the $(M+1)^{+\cdot}$ to $(M-15)^+$ peaks may be used as further proof of specificity. Since there are no interfering peaks in this technique, such monitoring was not needed routinely.

As shown in Figs. 5 and 6, no other ions of appreciable abundance appear in

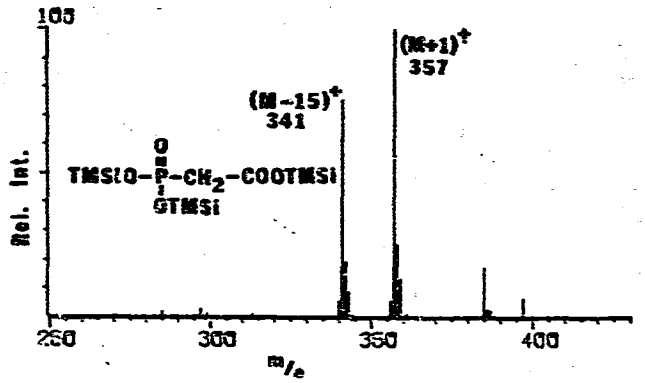

Fig. 5. Chemical ionization (methane) mass spectrum of the trimethylsilyl derivative of phosphonoacetic acid (PAA).

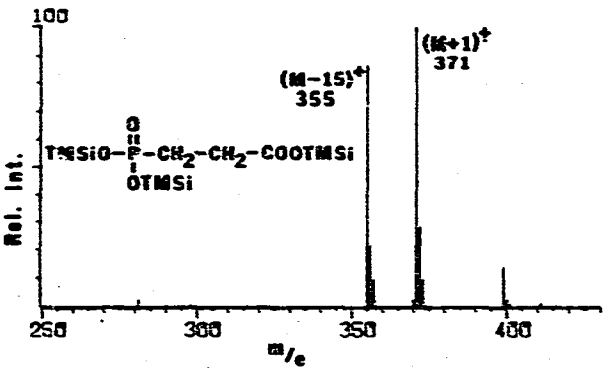

Fig. 6. Chemical ionization (methane) mass spectrum of the trimethylsilyl derivative of phosphonopropionic acid (PPA) used as internal standard.

the mass spectra of PAA and PPA. Ions with *m/e* higher than that of the protonated molecular ion represent the results of addition reactions by the methane reagent gas; such ions are customary in chemical ionization and are of no consequence in quantification.

Detection limits, quantification

The limit of detection of pure phosphonoacetic acid (as the TMS derivative) is 50 pg ($0.5 \cdot 10^{-12}$ mole) injected. The limit of detection of PAA in serum is 20 ng/ml using 0.2 ml sample size. When PALA is hydrolyzed to PAA, the detection limit for PALA is either 300 ng/ml (1.0 μM) when 0.5 ml initial sample is used, or 150 ng/ml (0.5 μM) when 1.0 ml initial sample is used (see Preparation of serum samples). The limit of detection is defined as the amount of substance needed to produce a peak/internal standard area ratio twice that of the blank. For reproducible quantification one needs to inject at least twice the amount corresponding to the limit of detection so that the computer could determine peak areas after appropriate background correction.

Quantification of PALA was accomplished with the aid of calibration curves. Normal serum samples spiked with an increasing amount of PALA (and a fixed amount of internal standard) were run and the ratios of the areas of PAA (from

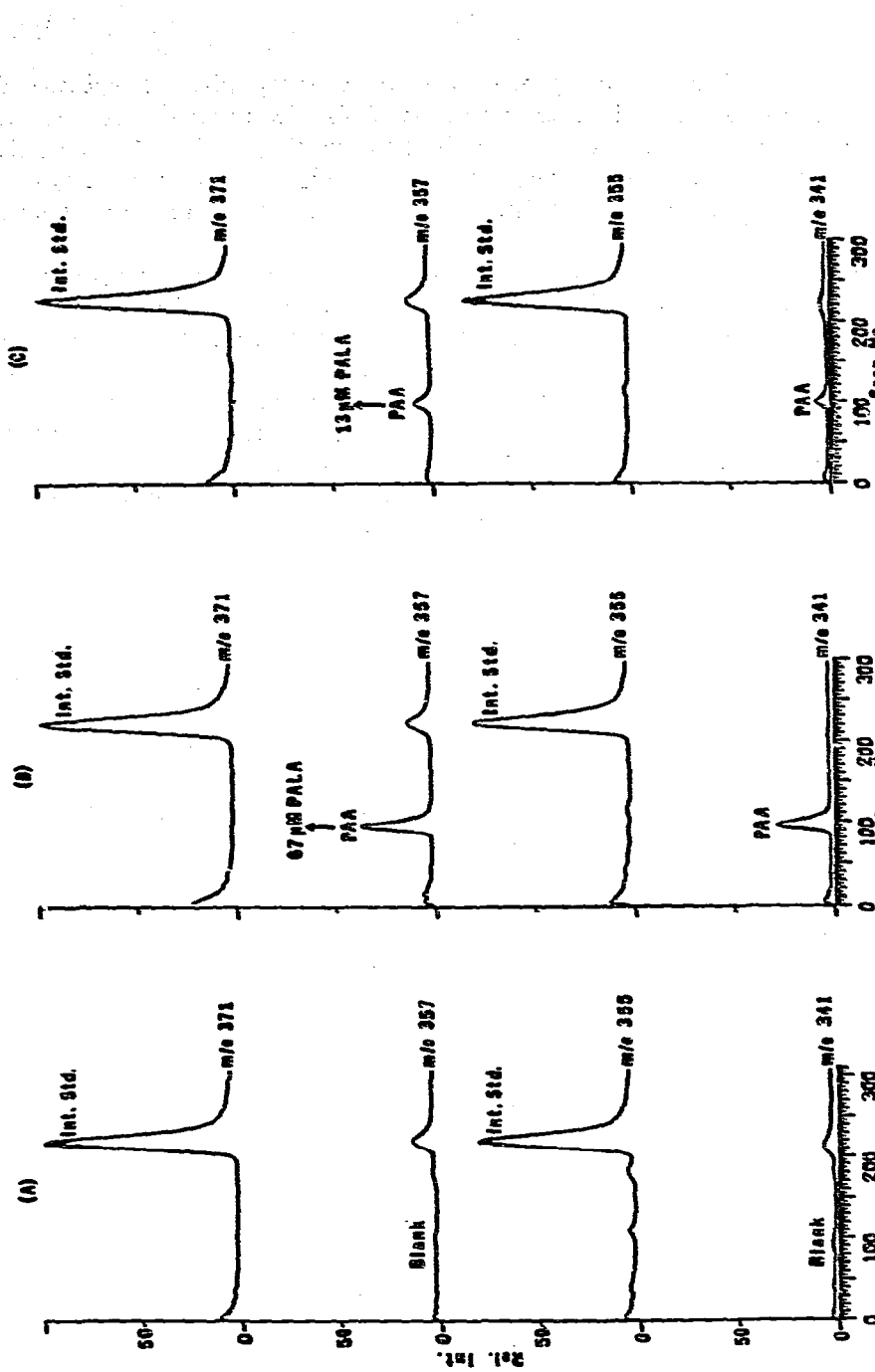

Fig. 7. Selected ion monitoring of phosphonoacetic acid (PAA) hydrolyzed from PALA in serum of patient receiving 1 g/m^2/24 h PALA by continuous infusion. (A) Zero time (pre-infusion); (B) end of infusion; (C) 4 h post infusion. Internal standard: phosphonopropionic acid (PPA).

PALA) and PPA, as determined by the computer, were plotted against the known amount of PALA initially added to a particular calibration sample. The calibration curves thus obtained were straight lines within a PALA concentration range of a factor of 100, and intercepted the Y-axis at or very near the origin. A new calibration curve was obtained for every set of patient samples analyzed.

Reproducibility measurements were made (n = 5) for each concentration point on the calibration curves. The coefficients of variation (defined as standard deviation divided by the mean × 100) were 5—10% in the 2.5—25 μg/ml PALA range, and 8—15% in the 0.5—5.0 μg/ml PALA range.

Concerning the analytical methodology, it is noted that very high levels of PAA may result in a "memory" effect, i.e., some material remains on the gas chromatographic column. Residual PAA can be removed with repeated flushings with the silylating reagent and concurrent increase of the temperature to 250°. Also, for best quantification the first few runs should be ignored when a new gas chromatographic column is employed.

Patient monitoring

Figs. 7 and 8 illustrate selected ion monitoring in patients. In Fig. 7 both the $(M+1)^{+\cdot}$ and $(M-15)^{+}$ peaks of PAA and PPA, respectively, were monitored. The gain in Fig. 8 is ten times that of Fig. 7; this is employed for low levels of PALA. (The computer normalizes to the internal standard.) The "zero time" or blank values represent samples taken prior to drug administration. The area of the blank which is seen only when high gain is used (Fig. 8A) corresponds to approximately 300 computer counts which is about the smallest area that could be quantified.

Fig. 7B shows the level of PALA at the end of a 24-h infusion period during which 1 g/m^2 of the drug was administered. The area of PAA corresponds to a concentration of 67 μM (20 μg/ml) of PALA which was the highest concentra-

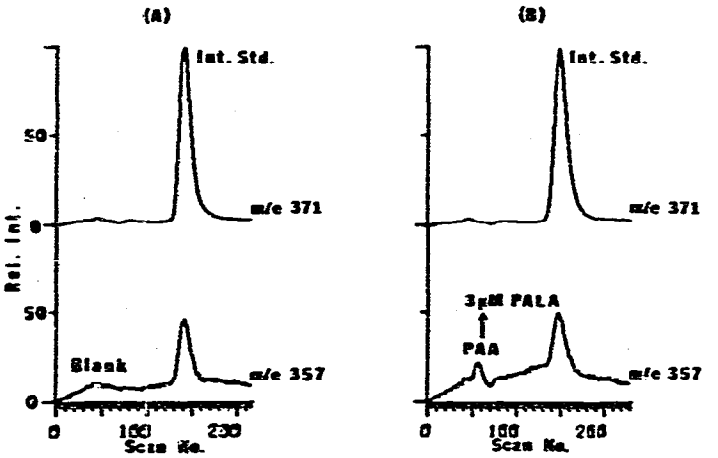

Fig. 8. Selected ion monitoring of phosphonoacetic acid (PAA) hydrolyzed from PALA in serum of patient receiving 4 g/m^2/5 days PALA by continous infusion. (A) Zero time (pre-infusion); (B) 12 h post infusion. Internal standard: phosphonopropionic acid (PPA). Gain is 10 times that of Fig. 7 for low-level PALA determinations.

tion measured during the infusion period. Decay of PALA levels commenced immediately upon termination of infustion. For example, 4 h later the concentration of PALA was 13 μM (Fig. 7C), and by 8 h the level of PALA was below detection limit. In contrast, when the same patient was given a dose of 4 g/m^2 for a 5-day period of infusion, at 8 h after infusion there was 5.3 μM PALA present, and 12 h after infusion there was still 3 μM present (Fig. 8B); it took 24 h to reach the limit of detection.

Details of the application of this technique to obtain pharmacokinetic data and correlations with clinical observations in Phase I trials in 37 patients were presented [13]; only a few relevant results are summarized here. In infusion studies the highest PALA levels (50—500 μM range) were observed at the end of the infusion period. Higher doses and/or longer infusion periods resulted in higher levels of PALA and longer decay curves. Serum drug levels decreased biexponentially in most cases, with an average first half-life of 100 min and an average second half-life of 8 h. There were at least two cases where PALA appeared to remain in the serum (at the 5 μM level) for at least 48 h after the infusion was terminated; this was accompanied by increased clinical toxicity.

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