CHROMBIO. 511

QUANTITATION OF THE ANTITUMOR AGENT N-(PHOSPHONACETYL)-L-ASPARTIC ACID IN HUMAN PLASMA AND URINE BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY—SELECTED ION MONITORING

ALAN R. BRANFMAN*, ROBERT J. BRUNI, YOON N. MERRILL and MARJORY CHADWICK

BioMolecular Sciences Section, Arthur D. Little, Inc., Acorn Park, Cambridge, Mass. 02140 (U.S.A.)

JOHN M. STRONG

Laboratory of Chemical Pharmacology, National Cancer Institute, National Institutes of Health, Bethesda, Md. 20205 (U.S.A.)

and

THOMAS J. ERVIN

Department of Medical Oncology, Sidney Farber Cancer Institute, Boston, Mass. 02115 (U.S.A.)

(Received September 17th, 1979)

SUMMARY

N-(Phosphonacetyl)-L-aspartic acid (PALA) is an antitumor agent which is currently under clinical study. A gas chromatography—mass spectrometry—selected ion monitoring assay procedure using [^{13}C]PALA as the internal standard has been developed for the quantitation of PALA in biological samples. Standard curves which related ion intensity peak height ratios (m/e 220/221) to PALA concentrations in plasma and urine were described by a non-linear least square analysis with correlation coefficients of R^2 >0.995 and >0.996, respectively. Over concentration ranges for PALA of 1—60 μg/ml of plasma and 1—160 μg/ml of urine the coefficient of variation from the fitted curve was 4—18%. This methodology has been used to quantitate PALA in human plasma samples in a study on the clinical pharmacology of the drug.

---

*To whom correspondence should be addressed.

INTRODUCTION

The potent antitumor agent [1] N-(phosphonacetyl)-L-aspartic acid (PALA, NSC 224131) is currently under clinical study by the National Cancer Institute. The drug is reported to act as a transition-state inhibitor of the enzyme aspartate transcarbamylase [2].

A sensitive and specific method of analysis for PALA in plasma was needed to provide clinical pharmacokinetic data. Recently two methods utilizing gas chromatography—mass spectrometry—selected ion monitoring (GC—MS—SIM) have been reported. The method of Strong and co-workers [3, 4] utilizes N-(phosphonacetyl)-L-glutamic acid as the internal standard, and that of Roboz et al. [5] involves quantitation of phosphonacetic acid after hydrolysis of PALA and utilizes phosphonpropionic acid as the internal standard [6]. Our method utilizes [^{13}C]PALA as internal standard. A preliminary account of this work was presented at a recent mass spectrometry conference [7].

EXPERIMENTAL

Materials

PALA disodium salt (77% purity, Lot No. HE 21-84-1) and [^{14}C]PALA (95% purity, sp. act. 32.9 μCi/mg, Lot No. 2333-32) were supplied by Drug Research and Development, Divison of Cancer Treatment, National Cancer Institute, U.S.A. Diazomethane in chloroform was prepared from N-methyl-N'-nitro-N-nitrosoguanidine (Aldrich, Milwaukee, Wisc., U.S.A.) in a diazomethane reaction vessel (Kontes, Vineland, N.J., U.S.A.). Ethereal diazomethane was generated from Diazald® (Aldrich). Saturated HCl in methanol was prepared by bubbling anhydrous HCl gas through methanol for about 30 min.

Synthesis of [^{13}C]PALA

[^{13}C]PALA (Fig. 1, IV) was prepared as follows from [^{13}C]phosphonacetyl chloride (Fig. 1, II) and L-aspartic acid dibenzyl ester (Fig. 1, III) according to the method of Starks Associates [8]. Diazomethane in diethyl ether (ca. 40 mmol) was added dropwise to a solution of 1 g (10.6 mmol) of [^{13}C]chloroacetic acid (1-^{13}C, 90% enriched) KOR Isotopes (Cambridge, Mass., U.S.A.) (Fig. 1, I) in 20 ml of diethyl ether. After 1 h stirring at room temperature, excess reagent and solvent were removed under a stream of nitrogen. The residual methyl chloro-[^{13}C]acetate was added under nitrogen to 7 ml of freshly distilled triethyl phosphite (b.p. 156°, Aldrich) and the solution was heated at reflux for 3 h [9]. The excess reagent was removed by short-path vacuum distillation and the remaining oil was vacuum distilled in a Kugelrohr bulb apparatus (Aldrich) to give 1.7 g (75%) of methyl diethoxyphosphinyl-[^{13}C]acetate, 80% purity by GC. The molecular ion observed at m/e 211 by GC—MS confirmed the structure as the desired intermediate. The methyl diethoxyphosphinyl[^{13}C]acetate (1.7 g, 7.9 mmol) was hydrolyzed in 13 ml of concentrated HCl by heating at reflux for 4 h to give 0.74 g (66%) of[^{13}C]-phosphonacetic acid after recrystallization from glacial acetic acid.

Fig. 1. Synthetic route to the internal standard, N-(phosphon-[^{13}C]acetyl)-L-aspartic acid.

A mixture of 0.74 g (5.2 mmol) of [^{13}C]phosphonacetic acid and 6 ml of thionyl chloride (Aldrich, freshly redistilled before use by maintaining the distillation flask temperature no higher than necessary for slow distillation) was stirred and heated to 50° for 16 h. Excess thionyl chloride was removed under vacuum and with the aid of dry benzene (distilled over sodium metal). The residual oil, [^{13}C]phosphonacetylchloride (Fig. 1, II) was dissolved in 5 ml of dry dioxane (distilled over sodium metal) and used directly for the next step.

L-Aspartic acid dibenzyl ester p-toluene sulfonate (3.05 g, 6.28 mmol), prepared according to the method of Zervas et al. [10], was dissolved in 20 ml of dry dioxane containing 1.75 ml (12.6 mmol) of triethylamine. The solution of [^{13}C]phosphonacetyl chloride (Fig. 1, II) in dioxane from the previous step was added dropwise with stirring. The temperature was maintained below 30° during the addition and then at room temperature for two additional hours. The insolubles were filtered off, the filtrate was concentrated in vacuo, and the oily residue was dissolved in methylene chloride. The methylene chloride solution was washed several times with water and then stirred with 45 ml of 0.7 N NaOH at 15° for 1 h and at 5° for 17 h. The aqueous layer was washed with methylene chloride and then with diethyl ether, concentrated to ca. 3 ml

and added dropwise to 30 ml of methanol with rapid stirring. The solid product was collected, redissolved in 40 ml of water and treated with 9 g of washed Bio-Rad AG 50W-X8, H⁺ form cation-exchange resin (Bio-Rad Labs., Richmond, Calif., U.S.A.). The solution of the free acid was neutralized to pH 8 with aqueous NaOH, concentrated, and the sodium salt precipitated in methanol as before. The vacuum-dried [¹³C]PALA tetrasodium salt (Fig. 1, IV) weighed 1.0 g (55%). Analysis: calculated for $C_5{}^{13}CH_6NNa_4O_8P \cdot 2.1\ H_2O$: C, 19.14; H, 2.69; N, 3.67; Na, 24.08; P, 8.11; H_2O, 10.08. Found: C, 19.22; H, 2.75; N, 3.59; Na, 23.89; P, 8.18; H_2O, 10.08.

Collection of clinical samples

PALA was administered to patients by a 5-day i.v. infusion. Blood and urine samples were collected at intervals up to 6 days after starting the infusion. Blood samples were transferred to heparinized tubes. Plasma was obtained by centrifugation and was frozen. Plasma and urine samples were stored at −20°.

Extraction of PALA from human plasma and urine

The standard curve samples were prepared in human plasma and urine over ranges of 1—60 μg/ml and 1—160 μg/ml, respectively. [¹³C]PALA (5 μg) was added to 0.2 ml of each clinical sample and PALA and [¹³C]PALA were extracted by the procedure reported previously [3]. PALA and [¹³C]PALA methyl esters were finally extracted into 100 μl of benzene, of which 2 μl were analyzed by GC—MS—SIM. The GC column was preconditioned with 5 injections of 2 μg of PALA tetramethyl ester. Standard curves were constructed with each set of patient samples.

Gas chromatography—mass spectrometry—selected ion monitoring

Quantitation was performed on a DuPont 21-491 mass spectrometer operated at 70 eV and interfaced with a Hewlett-Packard 5700A gas chromatograph. A DuPont 21-095 multiple specific ion detector was used for selected ion current measurements. A DuPont 21-094 data system was used for scanning mode data acquisition and processing. A 1.2 m × 2 mm I.D. glass column packed with 3% OV-17 on 100—120 mesh Gas-Chrom Q (Supelco, Bellefonte, Pa., U.S.A.) was used. The injection port, flame ionization detector, column, glass jet separator and ion source temperatures were set at 250°, 300°, 240°, 275° and 200°, respectively. The helium carrier gas flow-rate was 20—25 ml/min. A GC splitter and a micro needle control valve allowed selected passage of GC effluent into the mass spectrometer via the jet separator.

Standard curves by the NONLIN program

Standard curves were prepared by a least square fitting of the standard curve sample data to the equation:

$$R = (x + A)/(Bx + C)$$

where R is the ion ratio m/e 220 to m/e 221, x is the quantity of PALA added and A, B, and C are constants [11]. The least square analysis was carried out using a computer program called NONLIN [12]. Initial estimates of A, B, and C; limits on the possible values of A, B, and C; and the ion ratio versus concen-

tration data from the analyses of the standard curve samples are given as input to the program. A typical set of R values is presented in Table I.

TABLE I

TYPICAL SET OF R VALUES

x (μg/ml)	$R^\star$
0	0.143
10	0.606
20	1.095
30	1.427
40	1.801
50	2.158
60	2.477

*Each R value is a mean of nine replicate measurements.

The initial estimate of B is the ratio of the m/e 221 to m/e 220 ions in the mass spectrum of the PALA tetramethyl derivative and is calculated to be 0.13. C is the amount of internal standard ($[^{13}C]$PALA) multiplied by the isotopic purity. $[^{13}C]$PALA (5 μg) was added to 0.2 ml plasma samples and C is calculated to be 5 μg $\times$ (1.0/0.2)ml $\times$ 0.9 = 22.5 μg/ml. The $[^{13}C]$PALA used has a 90% isotopic purity. The sum of A and C is approximately the total amount of internal standard used and therefore $A = 5 \times 1.0/0.2 - C = 2.5$. The limits of A, B, and C are set in Table II.

TABLE II

THE LIMITS OF A, B, and C

	A	B	C
Initial estimate	2.5	0.13	22.5
Lower limit	0.5	0.05	10
Upper limit	5	0.3	30

Once the least square best fit values of A, B and C were found, they were used in the rearranged equation:

$$x = (RC - A)/(1 - RB)$$

to calculate the amount of PALA in each unknown plasma sample.

RESULTS AND DISCUSSION

The PALA extraction procedure [3] used here involved addition of the internal standard to samples of plasma or urine and precipitation of the protein and drug with acetone. The drug was extracted into tetrahydrofuran—methanol—hydrochloric acid, dried, and methylated with diazomethane. The recovery

of PALA from plasma and urine averaged 89.2 ± 2.8% (S.D.) and 102.8 ± 2.0% (S.D.), respectively, as determined with ^{14}C-labeled drug.

The methyl derivatives of PALA and [^{13}C]PALA co-chromatograph as a single GC peak (retention time 4 min) which allows simultaneous measurement of the monitored ions in the mass spectrometer. The mass spectral ions of interest were m/e 151 and 220 for PALA [13] and m/e 152 and 221 for [^{13}C]PALA methyl derivatives. Although measurement of m/e 151, the base ion, would result in the highest sensitivity, m/e 220 (50% relative intensity) was measured to avoid interference from a substance in plasma which chromatographed close to PALA and possessed an ion at m/e 151.

Standard curves which related ion intensity peak height ratios to PALA concentrations in plasma (Fig. 2) and urine (Fig. 3), were described by a non-linear least square analysis with correlation coefficients of R^2 >0.995 and >0.996, respectively. The values ± S.D. for the constants A, B and C were 2.905 ± 0.591, 0.092 ± 0.015 and 19.939 ± 0.948, respectively. Over concentration ranges for PALA of 1—60 μg/ml of plasma and 1—160 μg/ml of urine the coefficient of variation from the fitted curve was 4—18%. The applicability of using internal standards possessing single mass unit differences in selected ion monitoring analysis has been discussed recently by Horie and Baba [14].

Typical ion chromatograms from the analysis of plasma samples from a patient on PALA therapy are shown in Fig. 4. PALA concentrations in the plasma of a cancer patient being treated with PALA at 1800 mg/m^2/day by continuous i.v. infusion for 5 days are shown in Fig. 5. PALA concentrations varied between 15 and 40 μg/ml during the infusion and decreased to ca. 1 μg/ml, the lower limit of sensitivity of the method by 24 h after the end of the infusion. Further work is in progress on the quantitation of PALA in clinical urine samples and interpretation of the pharmacokinetic data will be reported in a future paper [15].
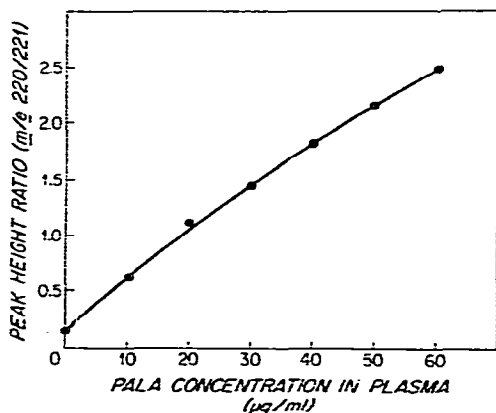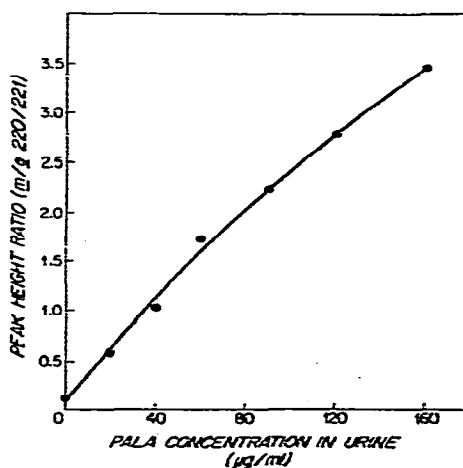

Fig. 2. Standard curve for PALA in plasma.

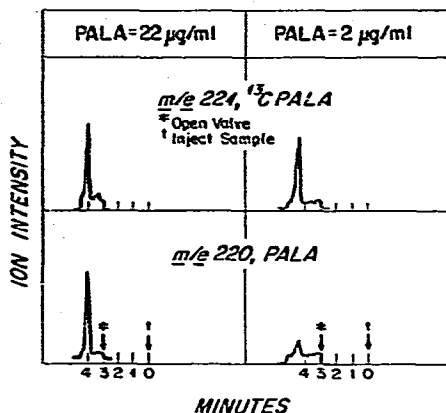Fig. 3. Standard curve for PALA in urine.

Fig. 4. PALA selected ion chromatograms.

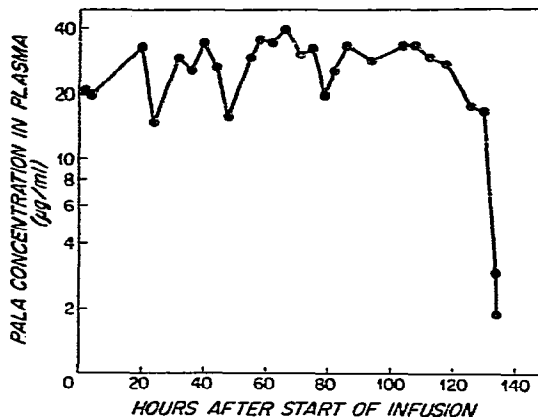Fig. 5. PALA concentrations in the plasma of a cancer patient.

REFERENCES

1   R.K. Johnson, T. Inouye, A. Goldin and G.R. Stark, Cancer Res., 36 (1976) 2720.
2   K.D. Collins and G.R. Stark, J. Biol. Chem., 246 (1971) 6599.
3   J.M. Strong, Y.E. Kinney, A.R. Branfman and R.L. Cysyk, Cancer Treat. Rep., 63 (1979) 775.
4   J.M. Strong, Y.E. Kinney, A.R. Branfman and C. Erlichman, 6th International Symposium on Mass Spectrometry in Biochemistry and Medicine, Venice, Italy, June 1979, Abst. 53.
5   J. Roboz, R. Suzuki, E. Rose and T. Ohnuma, 27th Annual Conference on Mass Spectrometry and Allied Topics, June 1979, Abstr. WAMOA3.
6   J. Roboz, R. Suzuki, G. Bekesi and R. Hunt, Biomed. Mass Spectrom., 4 (1977) 291.
7   R.J. Bruni, Y. Merrill, A.R. Branfman, J.M. Strong and T.J. Ervin, 27th Annual Conference on Mass Spectrometry and Allied Topics, June 1979, Abstr. MAMP9.
8   Starks Associates, Buffalo, N.Y., private communication via the National Cancer Institute (U.S.A.).
9   R.M. Davidson and G.L. Kenyon, J. Org. Chem., 42 (1977) 1030.
10 L. Zervas, M. Winitz and J.P. Greenstein, J. Org. Chem., 22 (1957) 1515.
11 B.H. Min, W.A. Garland, K.C. Khoo and G.S. Torres, Biomed. Mass Spectrom., 5 (1978) 692.
12 C.M. Metzler, A Users Manual for NONLIN, Upjohn Company, Kalamazoo, Mich., U.S.A., 1976.
13 A.R. Branfman, K.H. Valia and R.J. Bruni, J. Chromatogr., 151 (1978) 71.
14 M. Horie and S. Baba, Biomed. Mass Spectrom., 6 (1979) 63.
15 M. Chadwick, D.M. Silveira, J.A. MacGregor, A.R. Branfman, R.H. Liss and D.W. Yesair, Cancer Res., in press.