ELSEVIER

Journal of Chromatography B, 667 (1995) 247–257

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Quantitation of 4-hydroxycyclophosphamide/aldophosphamide in whole blood

L.W. Anderson[a], S.M. Ludeman[b], O.M. Colvin[b], L.B. Grochow[b], J.M. Strong[a,*]

[a]*Department of Clinical Pharmacology, US FDA, CDER, ORR, 4 Research Court, Rockville, MD 20850, USA*
[b]*Oncology Center, School of Medicine, Johns Hopkins University, Baltimore, MD 21287, USA*

First received 10 October 1994; revised manuscript received 30 December 1994; accepted 16 January 1995

Abstract

There is considerable interest in determining 4-hydroxycylcophosphamide/aldophosphamide (4-HO-CP/AP) blood levels in patients receiving the prodrug, cyclophosphamide (CP). Phosphoramide mustard (PM), the alkylating metabolite of CP, is relatively impermeable to cell membranes and it is generally believed that circulating intermediary metabolites, including aldophosphamide, the immediate precursor of PM, is transported by circulating blood to tumor tissue. Therefore, circulating 4-HO-CP/AP blood levels should more closely reflect the oncostatic and cytotoxic effects of CP than the parent drug. We have developed a gas chromatographic electron-impact mass spectrometric (GC–EIMS) method suitable for routine monitoring of 4-HO-CP/AP levels in whole blood over the range 0.085 μM (25 ng/ml) to 34 μM (10 μg/ml). The unstable metabolites were derivatized with O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine-HCl to form a stable aldophosphamide oxime derivative (PBOX). [$^2H_4$]PBOX was used as an internal standard. For clinical samples, tubes were prepared prior to blood drawing, which contained the derivatizing reagent solution and the internal standard. These solutions were stable for up to 3 months when stored at room temperature. Following addition of blood to the reaction tubes, PBOX formation was rapid and the resulting derivative was stable under these conditions for up to 8 days at room temperature. Application of the method was demonstrated by quantitating 4-HO-CP/AP blood levels in patients receiving 4 g/m^2 intravenous infusion of CP over a period of 90 min.

1. Introduction

The chemistry and metabolism of the prodrug, cyclophosphamide (CP), is complex and has been the object of numerous investigations [1–3]. Over the years, a major focus has centered on the metabolic pathway leading to formation of the alkylating moiety, phosphoramide mustard (PM), Fig. 1. CP is initially oxidized by cytochrome P-450 enzymes to form 4-hydroxycyclophosphamide (4-HO-CP), which equilibrates with the acyclic tautomer aldophosphamide (AP) [4]. AP undergoes β-elimination of acrolein to form the ultimate alkylating moiety, PM, which is relatively impermeable to cell membranes [5,6]. It is generally believed that circulating intermediary metabolites, including 4-HO-CP/AP, the immediate precursors of PM, are transported by blood to tumor tissue. Therefore, there is considerable interest in determining 4-

* Corresponding author.

SSDI 0378-4347(95)00036-4

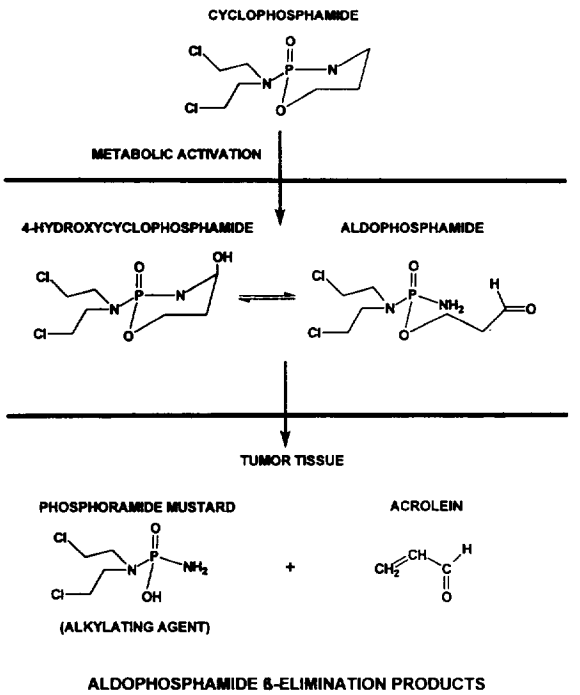

Fig. 1. Metabolic Scheme for CP transformation to PM.

HO-CP/AP blood concentrations in patients receiving CP because these circulating metabolites should more closely reflect the exposure of the tumor to the ultimate cytotoxic agent than the parent drug, CP.

Little data on circulating levels of 4-HO-CP/AP is present in the literature, despite the interest in these activated CP metabolites. This is primarily due to the lack of a convenient analytical method for quantitating these unstable CP metabolites. Previous methods include an indirect fluorometric method that measures acrolein eliminated during the conversion of 4-HO-CP/AP to PM and was used to monitor plasma 4-HO-CP/AP levels in patients receiving high dose (50 mg/kg) CP [7,8]. Wagner et al. [9] administered [^{3}H]CP to patients and determined 4-HO-CP/AP plasma and urine levels after reacting 4-HO-CP/AP with benzyl mercaptan followed by thin layer chromatography separation. 4-HO-CP/AP plasma levels in human plasma were measured by gas chromatography–chemical-ionization mass spectrometry (GC–CIMS) after stabilizing 4-HO-CP/AP as its cyanohydrin derivative, followed by silylation of the resulting derivative [10,11].

Central to developing a selective, useful quantitative method for 4-HO-CP/AP, is the rapid conversion of these unstable activated metabolites to a stable derivative. We have developed a method to quantitate 4-HO-CP/AP levels in whole blood by gas chromatography–mass spectrometry using electron-impact ionization (GC–EIMS). The derivatizing reagent, O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride, was reacted with 4-HO-CP/AP to form a stable AP oxime derivative (PBOX), Fig. 2.

One criterion considered during development of the method for clinical application was sample processing. To simplify the procedure, tubes were prepared prior to blood drawing, which contained the derivatizing reagent solution and internal standard. These solutions were stable for up to 3 months when stored at room temperature. Likewise, after adding the patient's blood to the tube and mixing, the resulting sample can be stored up to 8 days at room temperature before analysis.

2. Experimental

2.1. Synthesis of compounds

A detailed synthesis of the internal standard E/Z-$\beta,\beta,\beta',\beta'$-$^2H_4$-aldophosphamide O-(2,3,4,5,6-pentafluorobenzyl)oxime ([$^2H_4$]PBOX) is reported elsewhere [12]. In brief, bis-(2-chloro-2,2-dideuterioethyl)amine hydrochloride [13,14] was used to provide *cis*-$\beta,\beta,\beta',\beta'$-$^2H_4$-4-hydroperoxycyclophosphamide by analogy to the synthesis of unlabeled material [15]. An aqueous solution of the hydroperoxide was reduced with sodium thiosulfate and treated with O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride in methanol. The product was isolated by extraction and crystallized. Elemental analysis of the solid [found (theory)]: C, 35.15% (35.31%); H + ^{2}H as H (for this analysis, the molecular mass was calculated with 4 ^{2}H atoms but the instrumentation analyzed each deuterium as though it were hydrogen), 3.69% (3.61%); N,

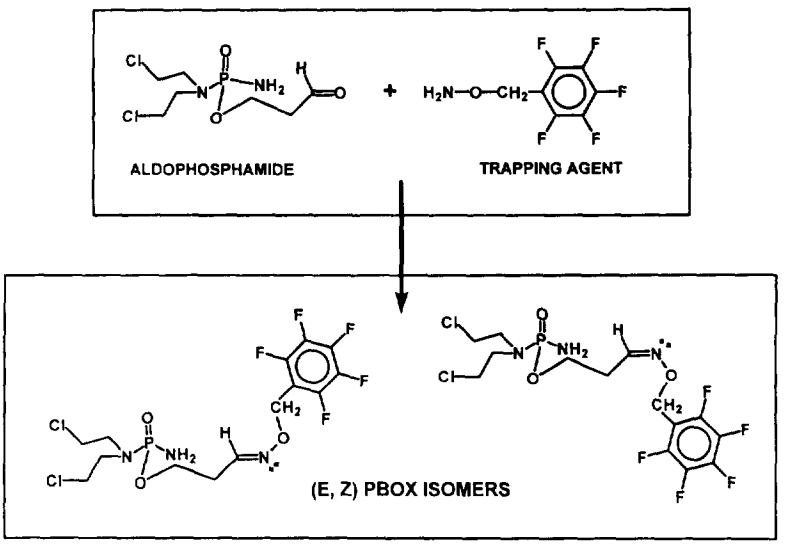

Fig. 2. Reaction of AP with O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine-HCl to form the stable E and Z isomeric oxime derivatives (PBOX).

8.72% (8.83%). Isotopic purity as determined by GC–EIMS was 0.5% $^{2}H_{0}$, 0.5% $^{2}H_{1}$, 0.5% $^{2}H_{2}$, 5.1% $^{2}H_{3}$, and 95% $^{2}H_{4}$.

Unlabeled E/Z-aldophosphamide O-(2,3,4,5,6-pentafluorobenzyl)oxime (PBOX) was synthesized as above using unlabeled *cis*-4-hydroperoxycyclophosphamide (4-HOOCP). The same product was obtained in reactions conducted under similar conditions but in the absence of sodium thiosulfate.

2.2. Nuclear magnetic resonance

^{1}H NMR spectra at 500 MHz and ^{31}P NMR spectra at 202.5 MHz were obtained on a Bruker MSL500 spectrometer. ^{1}H NMR chemical shifts were referenced to the internal standard, tetramethylsilane. ^{31}P NMR chemical shifts refer to a capillary insert of 1% H_3PO_4 in H_2O. As a lock signal, a small amount of $^{2}H_2O$ was added to samples for ^{31}P NMR spectral analyses.

2.3. Mass spectrometry

Positive ions (LSIMS) were obtained on a Model HP 5988 mass spectrometer (Hewlett-Packard, Palo Alto, CA, USA) equipped with a Cesium ion gun (Phrasor Scientific, Duarte, CA, USA). The primary ion beam was accelerated to 10 keV and samples were dissolved in a glycerol–3-nitrobenzyl alcohol (9:1, v/v) matrix.

GC–EIMS was performed on a HP 5970 MSD equipped with a HP 5980 gas chromatograph (Hewlett-Packard). The GC injector and transfer line temperatures were held at 275°C and 290°C, respectively. Compounds were separated on a 20 m × 0.25 mm I.D. DB-5 (0.1 μm film) capillary column (J and W Scientific, Folsom, CA, USA) using helium as the carrier gas at a column head pressure of 2 psi. After sample injection, the GC column was held at 165°C for 2 min and then ramped to 240°C at a rate of 4°C/min.

2.4. Blood sample processing

4-HO-CP/AP are unstable in human blood [10], therefore, blood samples must be processed immediately after blood drawing to form a stable derivative of AP. To facilitate the processing, tubes were prepared before blood drawing by adding 2 ml of acetonitrile, 1 ml of methanol, 1 ml of 2 *M* ammonium phosphate (pH 4.6), and 250 μl of a methanol solution containing O-

(2,3,4,5,6-pentafluorobenzyl)hydroxylamine (50 mg/ml) and the internal standard, [$^2H_4$]PBOX (16 μg/ml). Each tube was labeled with its tare weight, capped, and stored at room temperature until needed. At time of blood drawing, approximately 1 ml of whole blood was added to the previously prepared sample tube, the tube capped, mixed thoroughly, and stored at room temperature for at least 3 h to ensure maximum derivatization of AP. Tubes were reweighed to obtain the weight of whole blood added.

2.5. Isolation and silylation of PBOX and [$^2H_4$]PBOX

Blood samples were vortex-mixed, the cellular debris sedimented by centrifugation at 1000 *g* for 5 min, and the supernatant transferred to a clean glass tube. After addition of 1 ml of chloroform, the sample was vigorously vortex-mixed, 1.6 ml of the lower (chloroform) phase transferred to a glass injection vial, and the solvent removed under a stream of air. Silylation of the compounds was achieved after adding 250 μl of acetonitrile and 60 μl of N-*tert.*-butyldimethylsilyl-N-methyltrifluoroacetamide to the residue. Samples were reacted for 1 h at room temperature before analysis by GC–EIMS.

2.6. Analysis of 4-HO-CP/AP by GC–EIMS

Human whole blood standards were prepared containing 4-HOOCP, the synthetic precursor to 4-HO-CP/AP, at the following concentrations: 0.000, 0.034, 0.085, 0.171, 0.341, 0.853, 1.71, 3.41, 8.53, 17.1, and 34.1 μM. Aliquots (1 ml) of each blood standard were prepared as described and the silylated derivatives of PBOX and [$^2H_4$]PBOX injected onto the GC system for analysis. Ion clusters at *m/z* 241–248, *m/z* 252–255, and *m/z* 492–498 were monitored. The area ratio, *m/z* 241 (PBOX) to *m/z* 245 ([$^2H_4$]PBOX), was used for quantitation, and the other ion clusters monitored were used for qualitative identification of the AP derivatives. Peaks for both the E and Z isomers of PBOX and [$^2H_4$]PBOX were present in the chromatogram; however, only the later-eluting more abundant isomer was used for quantitation.

2.7. PBOX formation in human blood samples

Conversion of 4-HO-CP/AP to PBOX in whole blood samples was evaluated after adding known amounts of (4-HOOCP) to four individual pooled human blood samples containing the internal standard, [$^2H_4$]PBOX, and derivatizing solution. At selected times, three 1-ml aliquots were removed from each pooled blood sample, extracted, silylated, and PBOX concentrations determined by GC–EIMS.

2.8. Stability of PBOX derivatizing solution

A set of tubes was prepared for processing blood samples containing the derivatizing reagent and internal standard, [$^2H_4$]PBOX, and stored at room temperature. At specified times, 1-ml aliquots of blood containing 3.41 μM 4-HOOCP were added to three individual tubes in the set and analyzed for PBOX by GC–EIMS.

2.9. Stability of PBOX in derivatizing solution and whole blood

Because it is often inconvenient to immediately analyze clinical samples on a routine basis, the stability of PBOX in human whole blood was evaluated. 4-HOOCP was added to pooled whole blood to give a final concentration of 3.41 μM. Aliquots (1 ml) were placed in previously prepared tubes containing the derivatizing solution and internal standard as previously described. The tubes were vigorously mixed and stored at room temperature. At specified times, three samples were selected from the set and the PBOX concentration was determined by GC–EIMS as described.

2.10. Patients samples

Blood samples were obtained from patients receiving CP (4 g/m^2) by intravenous infusion over a 90-min period as part of induction therapy prior to bone marrow transplantation. Written

informed consent was obtained from each patient to provide samples of whole blood (30 ml total). Blood samples (1 ml) obtained at specified times from an indwelling central venous access were immediately added to prepared tubes containing the derivatizing solution and thoroughly mixed as described.

3. Results

3.1. Structural identification of PBOX and [$^2H_4$]PBOX

4-HO-CP/AP generated from 4-HOOCP was reacted with O-(2,3,4,5,6-pentafluorobenzyl)-hydroxylamine hydrochloride to form stable E and Z PBOX isomers, Fig. 2. The internal standard, $^2H_4$-PBOX, was synthesized as described. Structural identification of the oxime derivatives was confirmed by ^{31}P and ^{1}H-Nuclear Magnetic Resonance (NMR), and LSIMS.

Nuclear magnetic resonance

4-HO-CP/AP, produced by the sodium thiosulfate reduction of 4-HOOCP [15], was reacted with O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride to give the stable E and Z PBOX isomers (Fig. 2). The same product was obtained from solutions of 4-HOOCP, which was not pre-reduced (i.e. reactions conducted in the absence of thiosulfate). These syntheses, as well as that of the internal standard [$^2H_4$]PBOX, are described briefly in the Experimental section and in detail elsewhere [12]. Structural identification of the oxime derivatives was confirmed by ^{31}P and ^{1}H nuclear magnetic resonance (NMR) and LSIMS.

^{31}P NMR

The ^{31}P (202.5 MHz) NMR spectrum of PBOX (in water–methanol, ca. 1.4:1, v/v) displayed two signals in a ratio of 54:46 [± 3 (average deviation, $n = 5$)] at 19.4 and 19.3 ppm, respectively, relative to 1% H_3PO_4. The chemical shifts of these signals were consistent with those expected for the E and Z isomers of an acyclic oxime [16].

^{1}H NMR

The ^{1}H (500 MHz) NMR spectrum of the synthesized E and Z isomers of (PBOX) in $CDCl_3$ is shown in Fig. 3. Resonance-doubling for three types of protons was observed: the imino proton (CH = N, triplets), the benzylic protons (CH_2ON, singlets), and the $CH_2C=N$ moiety (apparent quartets). For various O-methyl oximes, it has been shown that the resonance for the imino proton in the E isomer occurs ca. 0.6–0.9 ppm downfield relative to that for the Z diastereomer [17,18]. Based on these reported chemical shift differences (as well as the expected greater favorability of the E isomer in terms of steric arguments), the triplets at 7.43 and 6.83 ppm were assigned, respectively, as the E and Z isomers of the aldophosphamide oxime. The signals which displayed resonance-doubling were used to determine an average E/Z product ratio of 56:44 [± 2 (average deviation, $n = 3$)] which, within experimental error, was the same ratio as that derived from the ^{31}P NMR data.

Mass spectrometry

A protonated molecular ion $[M+H]^+$, m/z 472, was observed in the PBOX LSIMS spectrum (data not shown). *tert.*-Butyldimethylsilyl derivatives of PBOX and the internal standard, [$^2H_4$]PBOX, were used for analysis of these compounds by GC–EIMS. Fig. 4 is a LSIMS spectrum of silylated PBOX, exhibiting a $[M+H]^+$ ion at m/z 586. The molecular ion cluster indicates the presence of two chlorine atoms and is consistent with a mono-substituted *tert.*-butyldimethylsilyl derivative of PBOX. Cleavage of silylated PBOX resulted in the ions observed at m/z 252 ($[A]^+$) and m/z 335 ($[BH]^+$). The *tert.*-butyldimethylsilyl PBOX derivative was analyzed by GC-EIMS and a representative total-ion chromatogram is shown in Fig. 5. The two peaks observed in the total-ion chromatogram with retention times of approximately 25 min. are consistent with the presence of E and Z isomers as observed in the NMR spectra of PBOX. EI mass spectra, obtained for these two peaks were qualitatively similar to each other and revealed the presence of ion clusters at m/z 492 containing 1 chlorine atom. It was postulated

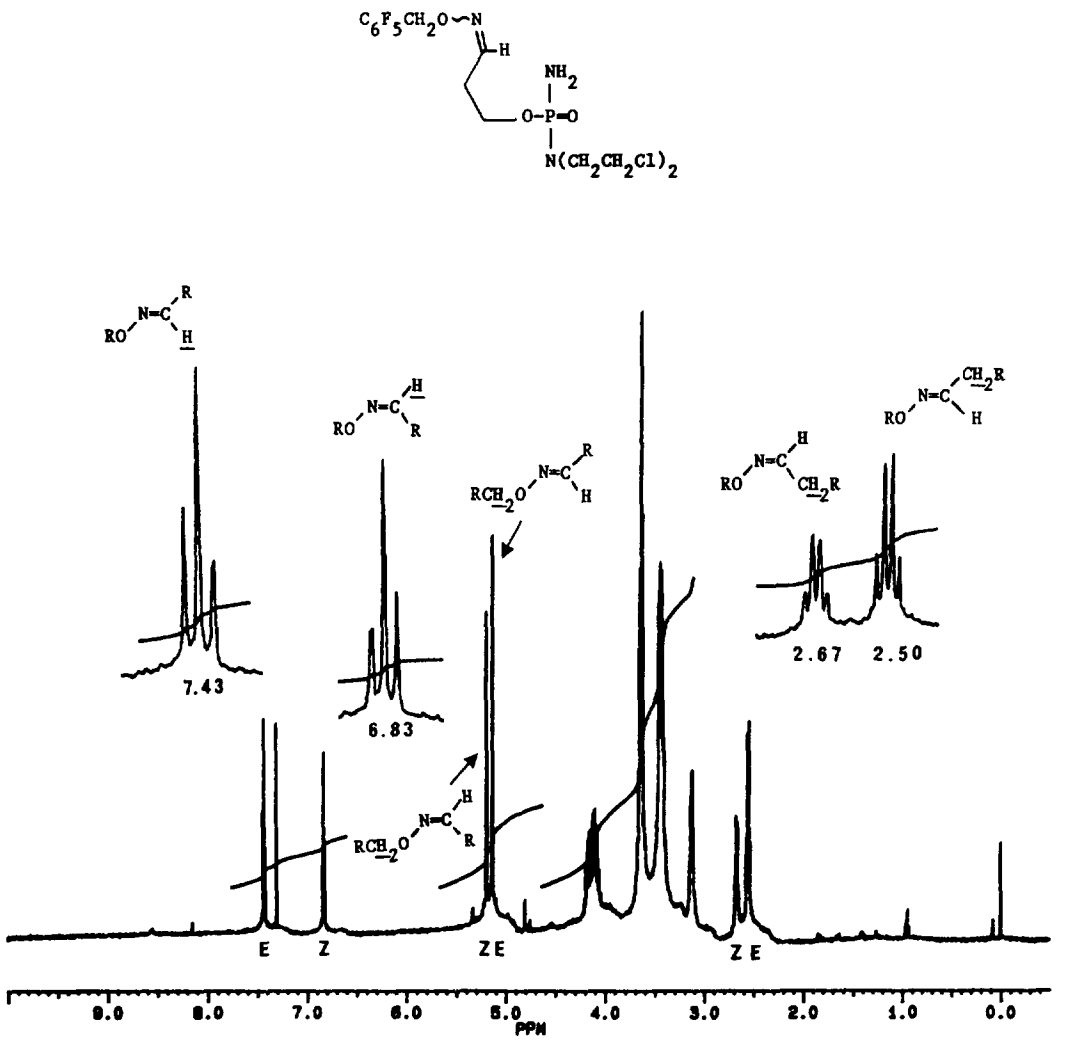

Fig. 3. ^1H (500 MHz) NMR of E/Z-aldophosphamide O-(2,3,4,5,6-pentafluorobenzyl)oxime (PBOX) in $CDCl_3$. Insets: expanded spectral regions showing resonances for the indicated protons of the E and Z isomers.

that a thermal decomposition product of silylated PBOX was formed in the injector port of the GC resulting in the structure shown in Fig. 6. Loss of C_4H_9 from this molecule (commonly observed for *tert.*-butyldimethylsilyl derivatives) would give rise to the ion cluster at *m/z* 492. To test this hypothesis, 4-hydroxyperoxyiphosphamide, a structural isomer of 4-hydroxyperoxycyclophosphamide, was reacted with O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride to form the oxime derivative of aldoiphosphamide. The *tert.*-butyldimethylsilyl derivative of this compound was analyzed by GC–EIMS. If our postulation were correct, then thermal decomposition of the silylated oxime derivatives of both CP and iphosphamide would be expected to produce the same molecule as shown in Fig. 7. Derivatized aldoiphosphamide peaks observed in the total-ion current tracing, Fig. 5, had retention times the same as those observed for PBOX formed from 4-HOOCP. Mass spectra obtained for these two peaks were identical to those observed for silylated PBOX. These results suggest that both 4-HOOCP and

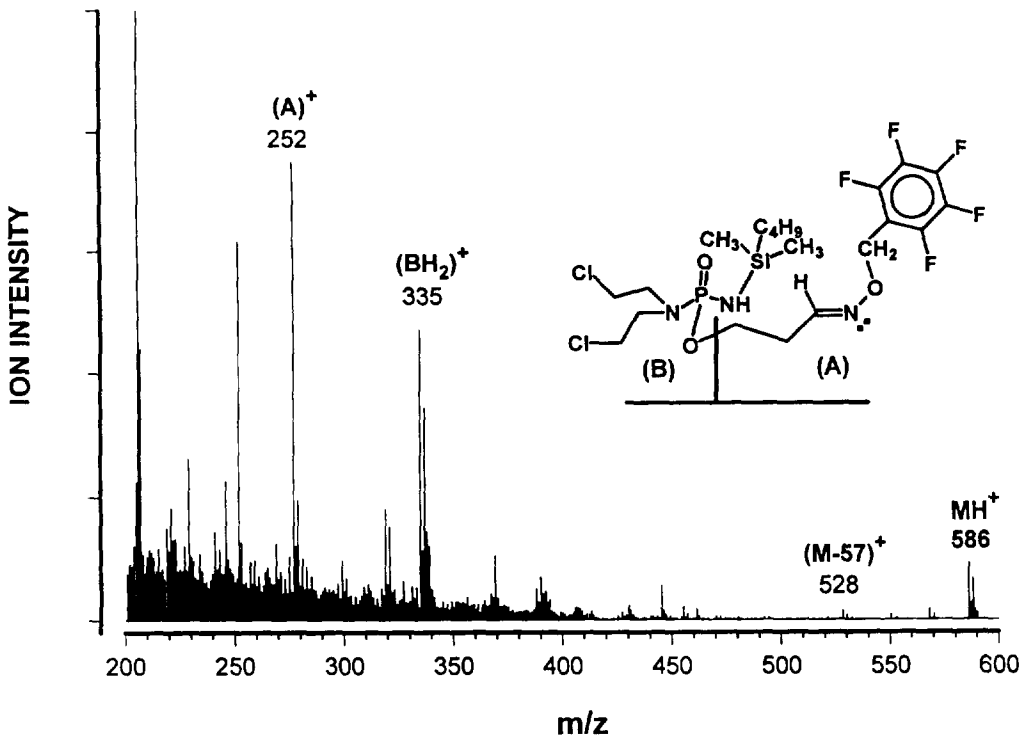

Fig. 4. Positive ion LSIMS spectra of *tert.*-butyldimethylsilyl PBOX.

4-hydroperoxyiphosphamide ultimately produced the same molecule when analyzed by our procedure, and support our hypothesis that a thermal degradation product of silylated PBOX was formed in the injector port. The fragment ions observed at *m/z* 241 and *m/z* 252 most likely represent the two halves of the molecule as shown in Fig. 4 and are similar to the fragmentation patterns observed in the LSIMS spectra obtained for silylated PBOX.

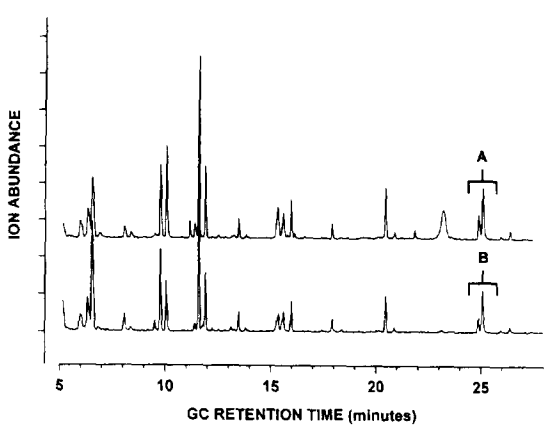

Fig. 5. Reconstructed total-ion chromatograms of (A) *tert.*-butyldimethylsilyl PBOX derived from 4-HOOCP, and (B) *tert.*-butyldimethylsilyl PBOX derived from 4-hydroperoxyiphosphamide.

3.2. Quantitation of 4-HO-CP/AP in whole blood

Validation of analytical method

Standard solutions of 4-HO-CP/AP formed from 4-HOOCP were analyzed by GC–EIMS as described. The area ratios, *m/z* 241 to *m/z* 245 were used to construct a standard curve shown in Table 1. The analysis was linear over 4-HO-CP/AP blood concentrations ranging 3 orders of magnitude from 0.034 μM to 34.1 μM. The slope and intercept of the regression line were $0.122 \pm 9 \cdot 10^{-4}$ S.E. and 0.007, respectively, with a correlation coefficient of 0.999. Expected

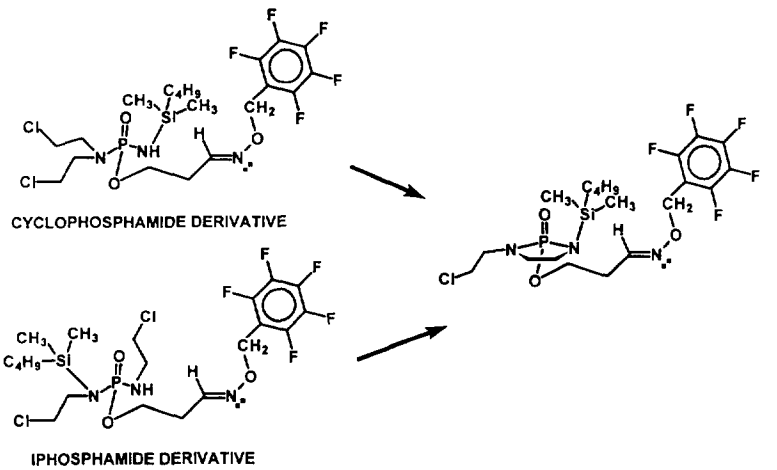

Fig. 6. Postulated gas chromatographic injection-port reaction occurring in the injection port yielding the same molecule for both CP and iphosphamide derived derivatives.

area ratios (theoretical mole ratios m/z 241 to m/z 245) based on the amount of 4-HOOCP and [$^2H_4$]PBOX added to each blood standard were compared with the experimentally measured ratios m/z 241 (PBOX) to m/z 245 ([$^2H_4$]PBOX) and are shown in Table 1.

PBOX formation in human blood

Whole blood samples containing 4-HOOCP and the derivatizing reagent were analyzed for AP at specified times after addition of the derivatizing solution. PBOX formation proceeded rapidly in the first hour (91.1 ± 6.3% S.D., 94.7 ± 4.6% S.D., 87.8 ± 6.2% S.D., and 96.1 ± 3.4% S.D. of the maximum amount of PBOX formed for four individual blood samples) and continued to slightly increase over the next 2 h. No additional PBOX formation was observed at 3 h following the addition of derivatizing agent. PBOX was stable for up to 8 days in the blood sample (Table 2), when stored at room temperature.

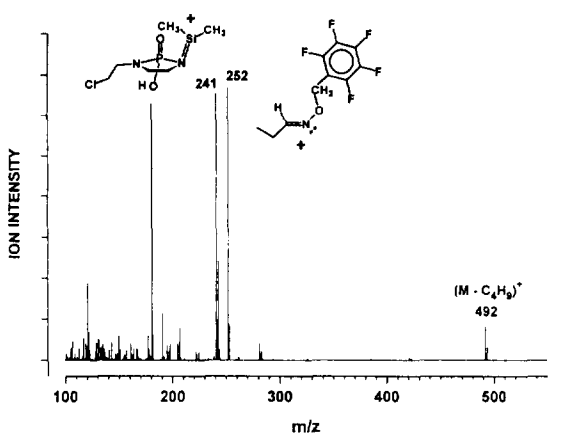

Fig. 7. EI mass spectrum of the *tert.*-butyldimethylsilyl PBOX with GC retention time of 25.05 min.

Table 1
Method validation

Standard (μM)	m/z 241 to m/z 245 ratio		
	Measured		Theoretical mole ratio
	Mean	S.D.[a]	
0	0.009	0.002	0.000
0.034	0.012	0.001	0.004
0.085	0.023	0.003	0.010
0.171[b]	0.034	0.000	0.020
0.341	0.049	0.002	0.040
0.853	0.106	0.001	0.101
1.70	0.221	0.008	0.201
3.41	0.359	0.001	0.403
8.53	1.06	0.041	1.01
17.0	2.16	0.043	2.01
34.1	4.15	0.127	4.03

[a] Three individual determinations at each concentration.
[b] Limit of quantitation.

Table 2
Stability of PBOX in whole blood

Days at room temp	Mean (μM)	S.D. (μM)	C.V. (%)
0.1	0.95	0.00	0.49
1.0	1.01	0.01	0.77
2.0	1.06	0.03	2.42
3.0	1.01	0.02	2.40
6.0	1.01	0.06	5.67
8.0	1.01	0.00	0.32

Sample processing and stability studies

A set of tubes prepared for processing blood samples obtained in the clinic and containing the derivatizing solution and internal standard, $[^2H_4]$PBOX, were stored at room temperature. At specified times, 1-ml aliquots of blood containing 3.41 μM 4-HOOCP was added to three individual tubes and analyzed for PBOX by GC–EIMS. Table 3 indicates that the solutions were stable and that no degradation of the internal standard, $[^2H_4]$PBOX, or the derivatizing reagent was detected over a period of 4 months.

Patient 4-HO-CP/AP blood levels

Blood samples were obtained at various times following start of infusion of CP and 4-HO-CP/AP levels were determined. A selected-ion tracing of extracts from a pre-dose blood sample and a blood sample obtained 8 h after start of infusion are shown in Fig. 8. The m/z 241 signal

Table 3
Stability of derivatizing reagent and internal standard in reaction tubes

Days at room temp	Mean (μM)	S.D. (μM)	C.V. (%)
0	3.16	0.60	19.1
9	3.56	0.44	12.4
10	3.72	0.21	5.8
15	3.52	0.36	10.2
24	3.82	0.27	7.0
30	3.78	0.08	2.1
41	3.86	0.49	12.8
127	3.86	0.29	7.6

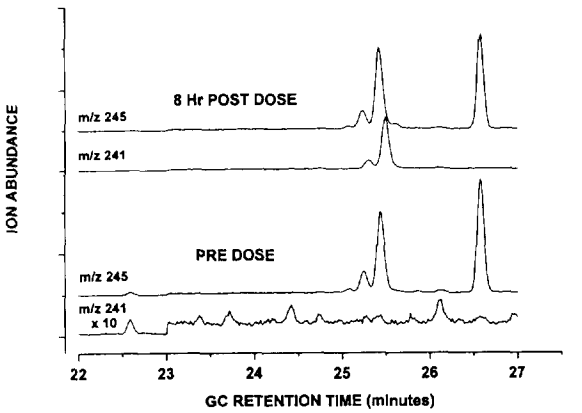

Fig. 8. Reconstructed ion chromatograms of patient samples before and 8 h after administration of CP; m/z 245 is the ion monitored for the internal standard, and m/z 241 is the ion monitored for patient produced PBOX.

in the pre-dose blood sample, amplified 10-fold, compared to other signals in the figure is free of interference over the elution time expected for the E,Z isomers of PBOX (t_R = 25.2–25.7 min). $[^2H_4]$PBOX concentration observed at m/z 245 was 8.47 μM and the 4-HO-CP/AP concentration was 5.99 μM in the 8-h sample.

4-HO-CP/AP blood levels determined in the patients are shown in Fig. 9. 4-HO-CP/AP levels reached a maximum concentration at 1.5 to 3.5 h ranging from 10 to 14 μM. Blood concentrations

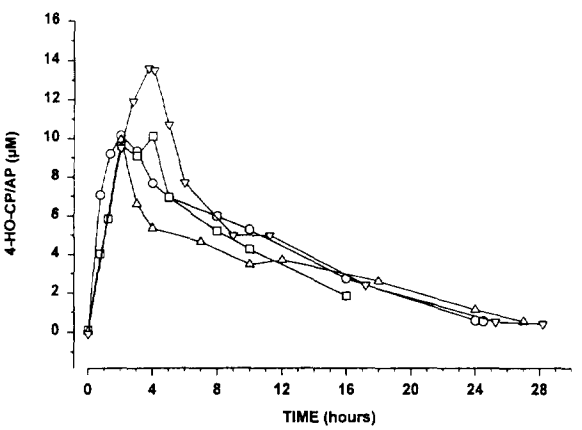

Fig. 9. Patient 4-HO-CP/AP blood levels during and after a 90-min i.v. push of 4 g/m^2 CP.

obtained in these patients at 24–28 h after start of infusion ranged from 0.50 to 0.70 μM.

4. Discussion

Analysis of 4-HO-CP/AP is complicated because of the inherent chemical instability of these CP metabolites. Estimates of 4-HO-CP/AP β-elimination rates in human plasma [10] and rat plasma [11], resulting in formation of acrolein and PM, were $t_{1/2} = 40$ min and $t_{1/2} = 7$ min, respectively. For clinical samples, we chose to quantitate 4-HO-CP/AP in whole blood in order to minimize the time between blood sampling and conversion of these unstable metabolites to the stable oxime. However, the method should be applicable to measuring 4-HO-CP/AP plasma levels as well. The method quantitates 4-HO-CP/AP in human whole blood over a concentration range of 0.085 μM (25 ng/ml) to 34 μM (10 μg/ml).

Previous GC–MS methods [10,11] used NaCN to "trap" 4-HO-CP/AP in human plasma followed by silylation of the resulting cyanohydrin derivative and quantitation by GC–CIMS. Similar to our method, both groups of investigators used 4-HOOCP, which is rapidly converted to 4-HO-CP/AP in H_2O as a reference standard for quantitation of 4-HO-CP/AP. These authors used *cis*-$\beta,\beta,\beta',\beta'$-$^2H_4$-HOOCP as an internal standard, which was added at the time of analysis. We synthesized a stable derivative, [$^2H_4$]PBOX, for use as an internal standard, which was added to the blood-sampling tubes and was stable at room temperature. The use of synthesized [$^2H_4$]PBOX as an internal standard allowed us to determine the conversion of 4-HOOCP added to whole blood to PBOX. Correlation of the expected (theoretical) m/z 241 (PBOX) to m/z 245 ([$^2H_4$]PBOX) ratio with those observed experimentally (Table 1) gave an intercept of 0.006, slope of 1.037 and a correlation coefficient, $r^2 = 0.999$. In addition, we chose to use EI ionization rather than CI so the method could be implemented on a simple low cost mass spectrometer.

The activated metabolites 4-HO-CP/AP are in pseudo-equilibrium with a number of molecular species as described by Zon et al. [16], including iminophosphamide and the reversible 4-thiol conjugates with 4-HO-CP. It has been hypothesized that these species could be present in circulating blood of patients treated with CP [1], but the concentration and importance of these compounds in human blood is yet to be established. We have generated several thiol conjugates of the activated metabolite in situ and have observed that these compounds are converted to PBOX in our analytical procedure. These observations indicate that AP levels determined in human blood would most likely include any molecular species in equilibrium with 4-HO-CP/AP.

Analytical methods developed for routine measurement of drug and metabolite blood or plasma concentrations in the clinic must be relatively simple and convenient with respect to sample processing following blood drawing. The use of O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride to derivatize the unstable AP metabolite of CP resulted in a stable oxime derivative. Derivatizing solutions containing the internal standard, [$^2H_4$]PBOX, were stable and could be prepared and stored at room temperature prior to use. In addition, we have demonstrated that PBOX was stable in whole blood for at least a week at room temperature, eliminating the need to immediately analyze the samples. Finally, we demonstrated the application of the method for quantitating 4-HO-CP/AP blood levels in patients receiving cyclophosphamide.

Acknowledgement

This investigation was supported in part by Public Health Service Grants 5-RO1-CA16783 (OMC) and 5-PO1-CA15396 (OMC) awarded by the National Cancer Institute (Department of Health and Human Services).

References

[1] E. Sladek, in G. Powis and R.A. Prough (Editors), *Metabolism and Action of Anti-Cancer Drugs*, Taylor and Francis, Philadelphia, PA, 1987, p. 48.

[2] M.J. Moore, *Clin. Pharmacokinet.*, 20 (1991) 194.
[3] L.B. Grochow and O.M. Colvin, *Clin. Pharmacokinet.*, 4 (1979) 380.
[4] T.K.H. Chang, C.L. Weber, L. Crespi and D.J. Waxman, *Cancer Res.*, 53 (1993) 5629.
[5] V.L. Boyd, M.F. Summers, S.M. Ludeman, W. Egan, F. Zon and J.B. Regan, *J. Med. Chem.*, 29 (1986) 1206.
[6] J.H. Boal, S.M. Ludeman, C.-K. Ho, J. Engel and U. Niemeyer, *Arzneim.-Forsch./Drug Res.*, 44 (1994) 84.
[7] T. Wagner, Heydrich, T. Jork, G. Voelcker and H.J. Hohorst, *J. Cancer Res. Clin. Oncol.*, 100 (1981) 95.
[8] N.E. Sladek, D. Doeden, J.F. Powers and W. Krivit, *Cancer Treat. Rep.*, 68 (1984) 1247.
[9] T. Wagner, G. Peter, G. Voelcker and H.J. Hohorst, *Cancer Res.*, 37 (1977) 2592.
[10] S.L. Pallante, L.B. Grochow, O.M. Colvin and C. Fenselau, presented at the *31st Annual Conference on Mass Spectometry and Allied Topics*, Boston, MA, May 8–13, 1983, p. 810.
[11] P.S. Hong and K.K. Chan, *J. Chromatogr.*, 495 (1989) 131.
[12] S.M. Ludeman, E.M. Shulman-Roskes, K.K.T. Wong, S.Y. Han, L.W. Anderson, J.M. Strong and O.M. Colvin, *J. Pharm. Sci.*, 12 (1995) in press.
[13] O.M. Colvin, R.B. Brundrett, M.-N.N. Kan, I. Jardine and C. Fenselau, *Cancer Res.*, 36 (1976) 1121.
[14] S.M. Ludeman, E.M. Shulman-Roskes, M.P. Gamcsik, T.G. Hamill, Y.H. Chang, K.I. Koo and O.M. Colvin, *J. Labelled Compd. Radiopharm.*, 33 (1992) 313.
[15] G.J. Zon, S.M. Ludeman, E.M. Sweet, W. Egan and L.R. Phillips, *Pharm. Sci.*, 71 (1982) 443.
[16] G. Zon, and S.M. Ludeman, J.A. Brandt, V.L. Boyd, G. Ozkan, W. Egan and K.-L. Shao, *J. Med. Chem.*, 27 (1984) 466.
[17] G.J. Karabatsos and N. Hsi, *Tetrahedron*, 23 (1967) 1079.
[18] G.J. Karabatsos and R.A. Taller, *Tetrahedron*, 27 (1968) 3347.