*Journal of Chromatography*, 495 (1989) 131–138
*Biomedical Applications*
Elsevier Science Publishers B V , Amsterdam — Printed in The Netherlands

CHROMBIO 4899

# ANALYSIS OF 4-HYDROXYCYCLOPHOSPHAMIDE BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY IN PLASMA

PHILLIP S HONG and KENNETH K CHAN*

*School of Pharmacy and Cancer Center, University of Southern California, 1985 Zonal Avenue, Los Angeles, CA 90033 (U S A )*

(First received February 21st, 1989, revised manuscript received June 12th, 1989)

## SUMMARY

A sensitive and specific method for the quantitative analysis of 4-hydroxycyclophosphamide/aldophosphamide has been developed using gas chromatography–mass spectrometry and a deuterium-labeled analogue as the internal standard The labile 4-hydroxycyclophosphamide/aldophosphamide and the internal standard in plasma were first converted into the more stable cyanohydrin adducts before extraction The isolated adducts were silylated and the products analyzed by gas chromatography–mass spectrometry The assay was found to be linear from 50 to 5000 ng/ml in plasma with a routine detection limit of 50 ng/ml The within- and between-run standard deviations at 100 ng/ml on eight replicate determinations were found to be 6 2 and 11 9%, respectively The extraction recovery was ca 80% This analytical method was used to evaluate the stability of 4-hydroxycyclophosphamide/aldophosphamide in fresh rat and pooled human plasma and to measure plasma 4-hydroxycyclophosphamide/aldophosphamide concentrations in the rat

## INTRODUCTION

Cyclophosphamide (CP), a widely used anticancer alkylating agent, requires microsomal activation for its action The initial metabolite, 4-hydroxycyclophosphamide (4-OHCP), exists in tautomeric equilibrium with the aldehyde form, aldophosphamide (AldP) under physiologic conditions [1–3] 4-OHCP/AldP undergoes chemical and/or enzymic degradation to release phosphoramide mustard (PM) and acrolein [4–6], the former of which has been considered as the ultimate cytotoxic metabolite of CP However, 4-OHCP/AldP has been considered to play a major role in transport of PM into cells [7,8] and the cytotoxic selectivity of the parent drug [9–13]

0378-4347/89/$03 50 

Although 4-OHCP/AldP has been identified for over a decade, its quantitation has not been accomplished until relatively recently due to the instability of the tautomers in biologic fluids A quantitative thin-layer chromatographic (TLC) method coupled to the use of radiolabeled CP was developed [14–20] However, the use of radiolabeled CP and the instability of the initial metabolites and their derivatives have limited its application especially in clinical studies An indirect method measuring acrolein fluorometrically following degradation of 4-OHCP/AldP has been used to assay 4-OHCP/AldP in plasma from rodents and patients treated with CP or 4-hydroperoxy-CP (4-OOHCP) [21–25], and the fluorometric method has been coupled to a high-performance liquid chromatographic method [26]. A crucial assumption for these methods is that acrolein of metabolic origin from CP in plasma prior to deproteinization is negligible, since acrolein, once formed, should quickly react with surrounding macromolecules Thus, plasma levels of acrolein generated from degradation of 4-OHCP following deproteinization was supposed to represent the true levels of 4-OHCP/AldP [25] This assumption, however, has not been clearly substantiated as yet [27]

Fenselau et al [28] isolated 4-OHCP/AldP from plasma as the cyanohydrin adduct and identified it using gas chromatography–mass spectrometry (GC–MS) after silylation. However, quantitation by GC–MS was not accomplished probably due to limited stability of the cyanohydrin adduct. This instability problem could be circumvented by the use of a stable isotope-labeled analogue, [$\beta$-$^2H_4$]4-OHCP/AldP, as the internal standard. Thus, in this report, a GC–MS assay method using a stable isotope dilution technique has been developed for the analysis of 4-OHCP/AldP in plasma The assay method was applied to monitor plasma 4-OHCP/AldP concentrations in vitro and in vivo

## EXPERIMENTAL

### *Standards and reagents*

4-OOHCP and [$\beta$-$^2H_4$]4-OOHCP were synthesized by the method of Takamizawa et al [29] CP and PM were supplied by Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD, U S A ) 4-OHCP and [$\beta$-$^2H_4$]4-OHCP were prepared by reduction of 4-OOHCP and [$\beta$-$^2H_4$]4-OOHCP, respectively, with sodium thiosulfate immediately before use [30] Semiconductor purity ammonia (99 999%) for chemical ionization was obtained from Matheson Gas (Cucamonga, CA, U.S.A.). For GC column packing (120 cm × 0 2 cm I D ), 3% OV-1 on 100–120 mesh Gas Chrom Q was purchased from Alltech Assoc (Deerfield, IL, U.S A ) Megabore fused-silica capillary columns were obtained from J & W Scientific (Rancho Cordoba, CA, U.S.A.). $C_{18}$ reversed-phase resin (Bondsil, Preparative grade, 40 $\mu$m) was purchased from Analytichem International (Harbor City, CA, U S A ) N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was

obtained from Pierce (Rockford, IL, U.S.A.). HPLC-grade methanol and methylene chloride were obtained from Curtin Matheson Scientific (Brea, CA, U S A ) Pooled human plasma was obtained from the Blood Bank of the Los Angeles County Hospital.

*Cyanohydrin adduct of 4-OHCP/AldP in plasma*

Plasma (100 $\mu$l) with 4-OHCP/AldP was placed in a cold (0°C) culture tube (100 mm×16 mm) containing 1 $\mu$g of [$\beta$-$^2H_4$]4-OHCP/AldP, the internal standard, dissolved in 100 $\mu$l of 0.9% saline solution. To the sample were added 200 $\mu$l of 1 *M* KCN–$NaHSO_3$ (pH 8) solution. The tube was vortexed and placed at room temperature for 30 min followed by freezing at −70°C

*Extraction and derivatization*

4-OHCP/AldP and the internal standard as the cyanohydrin adducts in plasma sample were extracted with 5 ml of methylene chloride. After centrifugation, the organic phase was separated and evaporated. The residue was derivatized with 40 $\mu$l of BSTFA at 120°C for 30 min before the GC–MS analysis.

*Gas chromatography–mass spectrometry*

A Hewlett-Packard 5985 A quadrupole mass spectrometer (Hewlett-Packard, Palo Alto, CA, U.S.A.) coupled with an HP 5840 gas chromatograph via an all-glass jet separator was used for the analysis. The temperatures of the transfer line, ion source, and jet separator were all maintained at 200°C, and the ionization current at 300 $\mu$A. For chemical ionization, the electron energy was set at 200 eV and ammonia was used as the reagent gas. A GC glass column (120 cm×0.2 cm I.D.) packed with 3% OV-1 on 100–120 mesh Gas Chrom Q was used The injection port was set at 220°C and the flow-rate of helium was 30 ml/min. The silylated and dehydrochlorinated 4-OHCP/AldP cyanohydrin adduct was eluted on the OV-1 column at 220°C isothermal with a retention time of 2.5 min. A similar retention time (2.2 min) was obtained under the same GC condition on a 15-m Megabore fused-silica capillary column (1 5 $\mu$m) with improved peak shape. Ions selected for GC–MS analysis were at *m/z* 412 and 416 for silyl derivatives of the dehydrochlorinated 4-OHCP/AldP cyanohydrin adduct [28] and the internal standard, respectively.

*Plasma 4-OHCP/AldP and PM analyzed as total PM*

4-OHCP/AldP in plasma (0.1 ml) was extracted along with PM on a Poly-Prep column (Bio-Rad, Richmond, CA, U.S A ) packed with 400 mg of $C_{18}$ resin using the published procedure [31] During the methanol elution and evaporation procedure, 4-OHCP/AldP was converted totally into PM. The GC–MS analysis of total PM, i.e. plasma PM plus PM derived from 4-OHCP/AldP, was accomplished by the method of Watson et al. [31] The true plasma

PM levels ($\mu M$) were calculated by subtracting plasma 4-OHCP/AldP levels from corresponding total PM levels.

*Stability of 4-OHCP/AldP in rat plasma*

4-Hydroxycyclophosphamide in rat and human plasma (0 4 m$M$) was incubated at 37°C A 0 2-ml aliquot of each sample was removed at 0, 2, 6, 10, 20, 40, 60, 70, 80, 100, and 120 min and placed in a culture tube containing 1 $\mu$g of the internal standard in 0 2 ml of 1 $M$ KCN–$NaHSO_3$ (pH 8) solution The sample was processed for 4-OHCP/AldP analysis as described before within three days Another 0.2-ml aliquot of each sample was removed and analysed for total PM All experiments were done in duplicate

*Animal protocol*

Male Sprague–Dawley rats (Simonsen, Gilroy, CA, U S A ) weighing 280–380 g were used and Purina Rat Chow (Purina and Wayne, Rialto, CA, U S.A ) and water given ad libitum The left jugular vein cannulation, drug administration, and blood sampling method were as described previously [32] Blood samples (0 5 ml each) were collected at 0, 2, 20, 30, 45, 60, 90, 120, 150, 180, 240, and 300 min into culture tubes (100 mm×16 mm) containing 20 $\mu$l of 1000 I.U heparin each and centrifuged at 2000 $g$ and 0°C for 1 min The loss of blood was replaced by administration of an equal volume of saline to the rat The separated plasma, 100 $\mu$l each, was placed in a culture tube (100 mm×10 mm) containing 1 $\mu$g of the internal standard in 100 $\mu$l of 1 $M$ KCN–$NaHSO_3$ and kept frozen until GC–MS analysis Another 100-$\mu$l aliquot was analyzed for total PM.

*Data analysis*

Regression and statistical analysis was accomplished on the PLOT4U program [33] (courtesy of Dr Michael B Bolger, School of Pharmacy, University of Southern California) using an IBM PC.

## RESULTS AND DISCUSSION

*Analysis of 4-OHCP/AldP*

*Stability of 4-OHCP/AldP* Since 4-OHCP/AldP is rather unstable in plasma [34], significant degradation may occur during sample manipulation The instability problem was circumvented by adding a deuterium-labeled analogue as the internal standard immediately following the separation of plasma This internal standard labeled with four deuterium atoms on the side-chain of 4-OHCP/AldP was assumed to degrade at the same rate with 4-OHCP/AldP during sample manipulation.

*Selected ions* The cyanohydrin adducts of 4-OHCP/AldP and [$\beta$-$^2H_4$]4-OHCP/AldP underwent dehydrochlorination [28] during silylation at 120°C

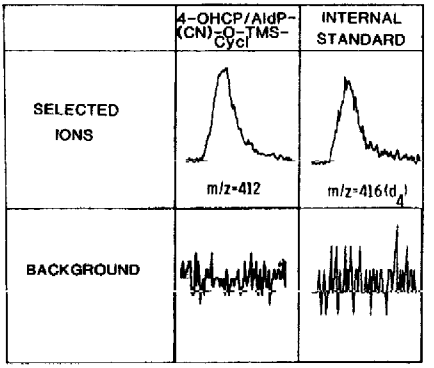


Fig 1 Selected-ion chromatograms of trimethylsilylated and dehydrochlorinated cyanohydrin adducts of 4-OHCP/AldP ($m/z$ 412) and the internal standard [$\beta$-$^2H_4$]4-OHCP/AldP ($m/z$ 416) derived from the plasma sample from the rat at 5 min following an intravenous bolus dose of 4-OHCP at 20 mg/kg Background counts ranged from 30 to 50 counts, and sample counts were larger than 300 counts

for 30 min The molecular ions for the silylated and dehydrochlorinated sample and the internal standard under GC–MS chemical ionization were at $m/z$ 412 and 416, respectively These ions were thus selected for GC–MS analysis and the ion chromatograms are shown in Fig 1. No interference ions were found in the blank human plasma extract, which is shown in the same figure.

*Linearity and sensitivity* The assay for 4-OHCP/AldP was linear from 50 to 5000 ng/ml in plasma monitored with a routine detection limit of 50 ng/ml The within- and between-run standard deviations at 100 ng/ml on eight replicate determinations were found to be 6 2% and 11 9%, respectively. The extraction recovery for 4-OHCP/AldP was ca 80%.

*Stability of 4-OHCP/AldP in plasma*

The degradation profile of 4-OHCP/AldP in rat plasma at 37 °C is shown in Fig 2 4-OHCP/AldP degraded monoexponentially with a mean half-life of 5 2 ± 0 2 min ($n=2$) PM was found to be the major degradation product and its concentrations peaked at 20 min and declined more slowly with a mean half-life of 52 ± 1 6 min In pooled human plasma, 4-OHCP/AldP also underwent monoexponential degradation with a mean half-live of 7 0 ± 0 5 min ($n=2$)

*Plasma concentration–time profiles of 4-OHCP/AldP following administration of synthetic 4-OHCP to the rat*

Plasma concentrations of 4-OHCP/AldP were found to decline monoexponentially as a function of time with a mean half-life of 6 9 ± 0 4 min ($n=2$) following administration of synthetic 4-OHCP/AldP (20 mg/kg) to the rat Details of the pharmacokinetic results will be published elsewhere [35]

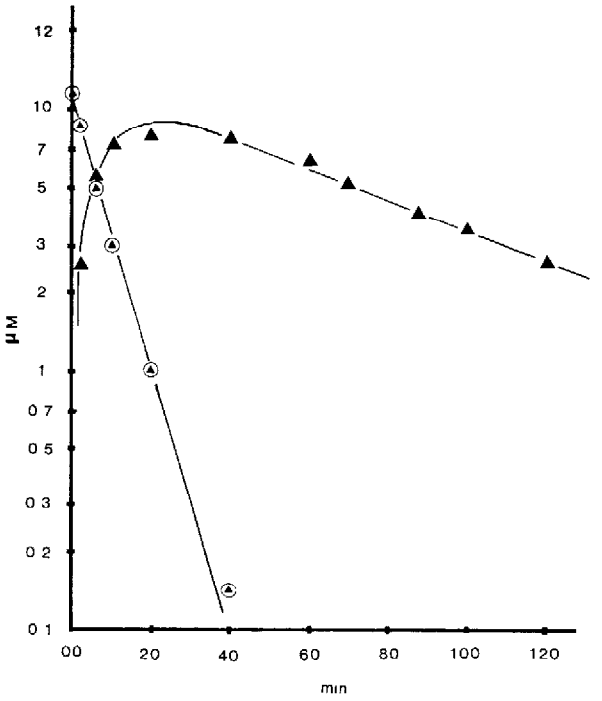


Fig 2 Stability profile of 4-OHCP/AldP (▲) in fresh rat plasma at 10 2 $\mu M$ incubated at 37 °C and concentration-time profile of PM (▲), the major degradation product of 4-OHCP/AldP The curves are regressed to equations $C_{4\ \mathrm{OHCP/AldP}} = C_0 \exp(-Kt)$ and $C_{\mathrm{PM}} = A[\exp(-Kt) - \exp(-k_d t)]$, where $C_{4\ \mathrm{OHCP/AldP}}$ = molar concentration of 4-OHCP/AldP, $C_{\mathrm{PM}}$ = molar concentration of PM, $C_0$ = initial concentration of 4-OHCP/AldP, $A$ = a hybrid coefficient with $A = C_0 K/(k_d - K)$, and $K$ and $k_d$ are apparent first-order rate constants for degradation of 4-OHCP/AldP and PM, respectively

*Plasma concentration-time profiles of 4-OHCP/AldP following administration of CP to the rat*

Following intravenous bolus administration of CP to the rat at a dose of 20 mg/kg, concentrations of 4-OHCP/AldP peaked at 20 min with a mean value of 3 4 ± 1 0 $\mu M$ ($n = 2$) and declined essentially in parallel with those of CP The peak concentration ($C_{\max}$) of 4-OHCP/AldP was found to be less than 25% of that of PM (20 ± 5 $\mu M$) as shown in Fig 3 Powers and Sladek [8] reported a $C_{\max}$ of 4-OHCP/AldP comparable with or higher than that of PM (ca 20–25 $\mu M$) following intraperitoneal administration of CP at 50 mg/kg to the rat The significantly higher ratio (> 1) of $C_{\max}$ of 4-OHCP/AldP to that of pM obtained from their report may be due to differences either in experimental design (administration routes, doses, etc ) or in analytical method

In conclusion, a quantitative method for the analysis of 4-OHCP/AldP in plasma was developed using GC–MS stable isotope dilution techniques This

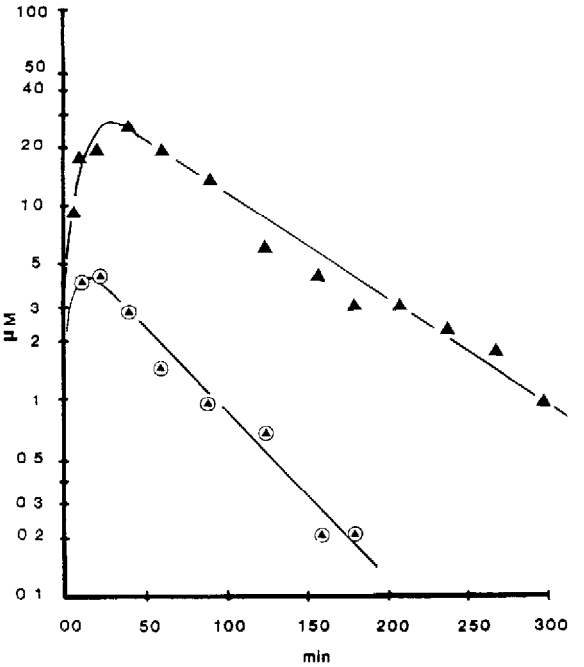


Fig 3 Plasma concentration-time profiles of 4-OHCP/AldP (▲) and PM (▲) following a single intravenous bolus administration of CP at 20 mg/kg to a rat The curves are regressed to a one-compartmental model equation for metabolites, i e $C_m = Ak_f[\exp(-k_m) - \exp(-Kt)]$, where $C_m$ = plasma concentration of 4-OHCP/AldP or PM, $A$ = a hybrid coefficient with $A = X_0/V_m(K - k_m)$, and $k_f$, $k_m$ and $K$ are apparent first-order rate constants for formation ($k_f$) and elimination ($k_m$) of a metabolite and the total elimination ($K$) of CP $V_m$ is the volume of distribution of 4-OHCP/AldP or PM, and $X_0$ the administered dose of CP

method was found to be sensitive and specific with a lower detection limit of 50 ng/ml The specificity for the analysis has been demonstrated by several specific requirements of the assay First, the sample compound possessed the necessary chemical characteristics to react with the added reagents to form the silylated cyanohydrin adduct which further underwent dehydrochlorination Secondly, the resultant product was eluted at the specific retention time and yielded the expected specific ion monitored Furthermore, no interference ion was experience in either blank plasma or plasma spiked with CP and other metabolites

ACKNOWLEDGEMENTS

This study is supported in part by PHS Grant CA 14089 and RO1 Grant 31693, both from the National Cancer Institute

REFERENCES


1 P J Cox, Br J Cancer, 28 (1973) 81
2 N E Sladek, Cancer Res , 33 (1973) 651
3 T A Connors, P J Cox, P B Farmer, A B Foster and M Jarman, Biochem Pharmacol , 23 (1974) 115
4 R A Alarcon and J Meienhofer, Nature (New Biol ), 233 (1971) 250
5 R F Struck, M C Kirk and M H Witt, Biomed Mass Spectrom , 2 (1975) 46
6 C Fenselau, M -N Kan and S Billets, Cancer Res , 35 (1975) 1453
7 H -J Hohorst, U Draeger, G Peter and G Voelcker, Cancer Treat Rep , 60 (1976) 309
8 J F Powers and N E Sladek, Cancer Res , 43 (1983) 1101
9 N E Sladek, Cancer Res , 33 (1973) 1150
10 P J Cox, B J Phillips and P Thomas, Cancer Res , 35 (1975) 3755
11 P J Cox, B J Phillips and P Thomas, Cancer Treat Rep , 60 (1976) 321
12 N Brock and H -J Hohorst, Z Krebsforsch , 88 (1977) 185
13 L Bielicki, G Voelcker and H -J Hohorst, J Cancer Res Clin Oncol , 105 (1983) 27
14 G Voelcker, T Wagner and H -J Hohorst, Cancer Treat Rep , 60 (1976) 415
15 T Wagner, G Peter, G Voelcker and H -J Hohorst, Cancer Res , 37 (1977) 2592
16 G Peter and H -J Hohorst, Cancer Chemother Pharmacol , 3 (1979) 181
17 T Wagner, D Heydrich, G Voelcker and H -J Hohorst, J Cancer Res Clin Ocol , 96 (1980) 79
18 R F Struck and D S Alberts, Cancer Treat Rep , 68 (1984) 765
19 R F Struck, D S Alberts, K Horne, J G Phillips, Y -M Peng and D J Roe, Cancer Res , 47 (1987) 2273
20 B E Doymer and N E Sladek, Cancer Res , 40 (1980) 174
21 G Voelcker, R Haeglsperger and H -J Hohorst, Cancer Res Clin Oncol , 93 (1979) 233
22 G Voelcker and R Haeglsperger, Arzneim-Forsch , 32 (1982) 639
23 N E Sladek, P C Smith, P M Bratt, N E Low, J F Powers and R F Borch, Cancer Treat Rep , 66 (1982) 1889
24 N E Sladek, J F Powers and G M Grage, Drug Metab Dispos , 12 (1984) 553
25 N E Sladek, D Doeden, J F Power and W Krivit, Cancer Treat Rep , 68 (1984) 1247
26 C A S Arndt, F M Balis, C L McCully, O M Colvin and D G Poplack, Cancer Res , 48 (1988) 2113
27 T Wagner, D Heydrich, T Jork, G Voelcker and H -J Hohorst, J Cancer Res Clin Oncol , 100 (1980) 95
28 C Fenselau, M N Kan, A Subba Rao, A Myles, O M Friedman and M Colvin, Cancer Res , 37 (1977) 2538
29 A Takamizawa, S Matsumoto, T Iwata, Y Tochino, K Katagiri, K Yamaguchi and O Shiratori, J Med Chem , 18 (1975) 376
30 G Zon, S M Ludeman, J A Brandt, V L Boyd, G Ozkan, W Eagan and K -L Shao, J Med Chem , 27 (1984) 466
31 E Watson, P Dea and K K Chan, J Pharm Sci , 74 (1985) 1283
32 P S Hong and K K Chan, Biomed Mass Spectrom , 14 (1987) 167
33 M B Bolger and K T Muir, in R Crosbie and P Luker (Editors), Proceedings of the 1986 Summer Computer Simulation Conference, Society for Computer Simulation, San Diego, CA, 1986, pp 49–53
34 C H Kwon, K Maddison, L Locastro and R F Borch, Cancer Res , 47 (1987) 1505
35 P S Hong and K K Chan, Drug Metab Dispos , in press