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Liquid chromatography–tandem mass spectrometric quantitation of cyclophosphamide and its hydroxy metabolite in plasma and tissue for determination of tissue distribution

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Abstract

Cyclophosphamide (CP) and its metabolite, hydroxycyclophosphamide (OH-CP) have been quantitated in mouse plasma and tissue by derivatization combined with liquid chromatography–tandem mass spectrometry (LC–MS–MS). The derivatization was conducted immediately upon sample collection, to trap the OH-CP metabolite intermediate prior to further conversion to phosphoramide mustard or other reaction products. This simple and straightforward derivatization procedure, combined with sample extraction via protein precipitation, allowed quantitation of CP and the oxime derivative of OH-CP in plasma for concentrations ranging from approximately 12.5–3333 ng/ml, and in spleen tissue for concentrations of 1250–50 000 ng/g. The short cycle time (2.5 min) of the LC–MS–MS method allowed high throughput analysis with minimal matrix interference. Mouse plasma levels were quantitated for doses of 40, 65 and 120 mg/kg; spleen concentrations were determined for mice dosed at 120 mg/kg. The CP and oxime plasma levels correlated well with dose amounts. The CP levels in the spleen and plasma were similar while the oxime levels in the spleen were significantly lower than the plasma.

Keywords: Cyclophosphamide; Hydroxycyclophosphamide

1. Introduction

Cyclophosphamide (CP) or cytoxan is a member of the nitrogen mustard subclass of anticancer drugs, and has a multi-faceted mechanism for rendering selective cytotoxicity. This primarily is due to the array of chemical and continued metabolic reactions of the initial 4-hydroxycyclophosphamide (OH-CP) metabolite formed from CP by cytochrome P_{450} enzymes. As shown in Fig. 1, it is widely believed that the continued breakdown via a β-elimination reaction of aldocyclophosphamide (ALDOCP), the tautomer of OH-CP, results in the release of acrolein and the cytotoxic phosphoramide mustard (PM), which acts as a DNA-alkylating agent. Through its involvement in DNA interstrand crosslinking, PM is

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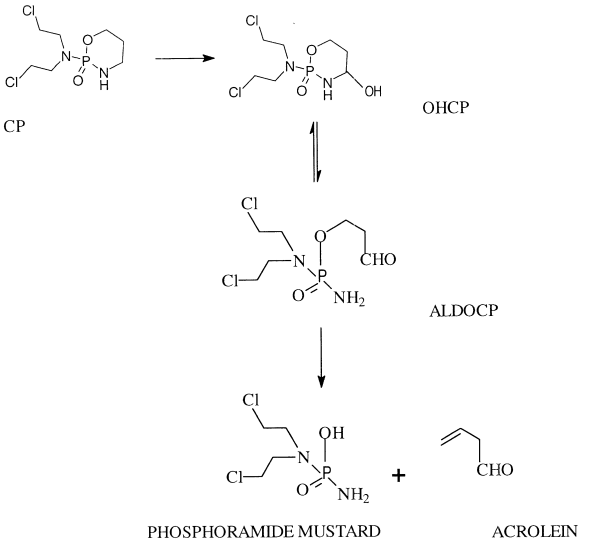

Fig. 1. CP and its metabolites resulting from cytochrome P_{450} mediated hydroxylation.

believed to prevent DNA replication and promote cell death [1]. In addition to the pathway shown in Fig. 1, other metabolic reactions produce several varied byproducts from CP. The complexity of CP's metabolism, pharmacokinetics and bioanalysis has been extensively reviewed in a recent issue of *Current Pharmaceutical Design* [2].

In order to draw a pharmacokinetic/pharmacodynamic (PK/PD) relationship between the amount of CP dosed and its activity, it is desirable to measure the plasma and tissue distribution of both the active drug (PM) and the prodrug CP. Analytically it is very challenging to quantitate phosphoramide mustard and acrolein, which are highly toxic and unstable compounds. A more practical approach is to quantitate the intermediate OH-CP, which is trapped using derivatization procedures immediately upon its formation in the biological matrix. Trapping of OH-CP is essential in plasma because of its instability ($t_{1/2}$=6 min) due to plasma enzymes [3]. Derivatization procedures with reagents such as *p*-nitrophenylhydrazine, potassiumcyanide, and 4-aminophenol/hydroxylaminehydrochloride have been used to stabilize these short-lived species [4–6]. These neither simple nor practical derivatization procedures complicate the analytical technique, and frequently require multiple extractions for analytes from the same sample. However, one of the derivatization procedures using *O*-methylhydroxylamine (OMHA), in which the ALDOCP/OH-CP is stabilized by forming an oxime of the aldehyde functionality, is relatively straightforward [7]. This simple derivatization procedure has been successfully used in clinical trials to immediately capture the OH-CP metabolite by drawing blood into tubes that contain the derivatizing reagent [8].

A recent review describes several existing analytical techniques to quantitate CP and many of its metabolites from various matrices [9]. Techniques such as gas chromatography (GC), high-performance liquid chromatography (HPLC), nuclear magnetic resonance spectroscopy (NMR), GC–mass spectrometry (MS), liquid chromatography–mass spectrometry (LC–MS) and liquid chromatography–tandem mass spectrometry (LC–MS–MS) have been used to measure CP and its metabolites in matrices like plasma, urine, blood, serum, human liver microsomes, S-9 liver fractions, and pharmaceutical dosage forms. A recently reported LC–MS–MS method has been used to quantitate only the prodrug CP [10]. However, no assays to date have reported the analysis of these compounds in any kind of tissue. The current method was developed in preparation to meet the analytical challenge of quantitating the amount of CP and OH-CP in both plasma and tumor, using spleen as a model tissue. Spleen was the tissue of choice since it is highly vascularized, allowing for a high degree of tissue distribution of systemically circulating drugs.

It has been demonstrated that the PK/PD relationship based only on the exposure of CP is not more predictive of the pharmacological effect than the amount of drug dosed [11]. This is not surprising since CP metabolites in the form of PM, not CP itself, are pharmacologically active. Therefore, it is important to measure the metabolite OH-CP which delivers the final cytotoxic agent phosphoramide mustard to the cells. The present work describes a LC–MS–MS assay with multiple reaction monitoring (MRM), for the measurement of both CP and OH-CP in the form of an oxime derivative. LC–MS–MS using MRM is more specific and sensitive than, single-ion monitoring (SIM), the technique used in a previously reported LC–MS assay [8]. More sensitive and selective determination of plasma and tissue

concentrations should be valuable in obtaining a better estimate of the PK/PD relationship and dose-related efficacy at the site of action, i.e., tumors in anti-cancer therapy.

2. Experimental

2.1. Preparation of dosing formulations

The solution of CP (MeadJohnson, USA) (120 mg/kg of mouse body mass) was prepared by adding 0.9% sodium chloride (Baxter Healthcare, USA). The lower dosages were prepared by making dilutions from the highest dosage. The solution was prepared immediately before use.

2.2. Animal studies

Two studies were performed. Spleen samples were analyzed for drug levels in experiment 1. For experiment 2, the plasma samples were analyzed. For both studies, 39 female nude mice (Crl:NU/NU-nuBR, Charles River Labs., Wilmington, MA, USA) were randomized and divided into four groups: three mice into the control group, 12 mice into each of the three drug dosage groups (120, 65, and 40 mg/kg). A 0.2-ml volume of of CP was administered intravenously (i.v.) on the basis of group average mass.

2.3. Plasma collection

Blood samples were taken by cardiac puncture from three mice per treatment group at 30 min, 1, 2, and 4 h post dose using a 1-ml syringe and 27 gauge needle that contained enough 0.5 *M* EDTA, pH 8.0 (Gibco, Grand Island, NY, USA) to fill the needle. From each animal, 0.5 ml of blood was placed into 1.5-ml microcentrifuge tubes, which contained 50 μl of 15 mg/ml OMHA. The samples were gently hand mixed by inverting the microcentrifuge tube 3–4 times. After mixing, the tubes were kept in an ice bath, until centrifugation at 14 000 rpm, at 4°C for 5 min. After centrifugation, the plasma was transferred to a 1.2-ml polypropylene tube, capped, and incubated in a 50°C water bath for 20 min. After incubation, the samples were frozen at −70°C.

2.4. Spleen collection

The spleens were removed, weighed, and placed into an 8-ml screw cap vial and quickly frozen in liquid nitrogen. For processing, ice cold water was added at 1 ml/100 mg of spleen mass and 15 mg/ml OMHA was added in a 1:10 (v/v) ratio to the spleen–water mixture. (For example: for a spleen mass of 130 mg, 1.3 ml ice cold water, 143 μl of 15 mg/ml OMHA were used). Using a Polytron mixer (Brinkmann, Model PT 10/35, 7 mm diameter obtained from VWR Scientific Products, USA with a foam reducing generator for 0.5–1.5 ml samples), the spleen was homogenized with water and OMHA. This homogenate was incubated in a 50°C water bath for 20 min. After incubation, the samples were frozen at −70°C.

2.5. Reduction and derivatization reactions

One mg/ml of Peroxycyclophosphamide (PCP) (Albany Molecular Research, USA), in methanol (Burdick & Johnson, USA) was reduced to 4-OH-CP with a threefold molar excess of sodium thiosulfate (Sigma, USA) in warm acetate buffer (100 m*M*, pH 5.7) at 37°C. A 10-μl aliquot of the reduction mixture was spiked into 490 μl of blank plasma or spleen homogenate to obtain a final concentration of 1000 ng/ml (plasma) or 10 000 ng/g (spleen) of 4-OH-CP. This stock solution was serially diluted with blank plasma or spleen homogenate to obtain the lower concentration standards. A 250-μl aliquot of each standard was treated with 25 μl of 15 mg/ml of OMHA at 50°C in a water bath for 20 min to obtain the oxime of 4-OH-CP. For plasma samples, OH-CP oxime standard concentrations were estimated at 3.1, 12.5, 125, 250, 500, 1000 and 3333 ng/ml. The OH-CP oxime standard concentrations were estimated at 125, 1250, 2500, 5000, 10 000 and 50 000 ng/g.

Appropriate volumes of 10 μg/ml and 1 μg/ml CP stock solutions were spiked into each of the serially diluted plasma standards prepared for OH-CP (as described previously) to obtain a final concentration of CP of 12.5, 125, 250, 500, 1000, and 3333 ng/ml in these standards. For spleen samples, cyclophosphamide standard concentrations of 1250, 2500, 5000, 10 000, and 50 000 ng/g were used. A

20-μl volume of the internal standard (I.S., synthesized in the laboratory) at a concentration of 2000 ng/ml was added to each of the standards.

Volumes (250 μl) of plasma and spleen standards were treated with 750 μl of acetonitrile to precipitate the proteins. The samples were vortex-mixed, and centrifuged at 3000 rpm for 15 min. A 500-μl volume of the supernatant was removed using a Tomtec Quadra 96 Model 320 workstation (Tomtec, Hamden, CT, USA) and dried under heated nitrogen using a 96 EvapArray APPARATUS (Porvair Sciences, distributed by Jones Chromatography, Lakewood, CO, USA). The evaporated residues were reconstituted with 25 μl of acetonitrile–water (50:50).

2.6. Sample preparation

A 20-μl volume of the I.S. (2000 ng/ml) was spiked into each of the derivatized samples. A 750-μl volume of acetonitrile was added to the samples. The samples were extracted using the same procedure as the standards.

2.7. Instrumentation

MS–MS experiments were performed on an Ultima system (Micromass, UK) in the positive ion electrospray mode, outfitted with a quaternary solvent delivery system and autosampler (Perkin-Elmer series 200, Norwalk, CT, USA). The capillary voltage was 3.4 kV, the drying gas was nitrogen and the collision gas was argon. Precursor to product transitions of the analytes at dwell times of 0.250 ms, in MRM mode are given in Table 1.

The LC system consisted of a YMC C_{18} basic column (5 μm particles, 50 mm×2.0 mm I.D., YMC, Milford, MA, USA). The mobile phase was composed of acetonitrile–0.1% formic acid (40:60) at a flow-rate of 200 μl/min. Injection volumes of 3.0 μl were utilized. The retention times for CP, OH-CP oxime, and I.S. were 1.1, 1.2 and 1.5 min, respectively. MS–MS data were processed using Masslynx software version 3.4 from Micromass and pharmacokinetic data processing was performed using Watson 6.1.

3. Results and discussion

Developing an analytical method for sample analysis to quantitate CP and the oxime of OH-CP involved: (1) adapting and optimizing the appropriate reduction reaction for PCP to OH-CP, and determining the MS–MS profile for the compounds, (2) adapting and optimizing the appropriate derivatization of OH-CP to oxime and determining the MS–MS profile for the oxime derivative, (3) developing a single LC method suitable for all compounds, (4) designing a sample processing strategy (plasma and tissue), (5) generating characterization curves for CP and the oxime of OH-CP to determine linearity and detection limits.

3.1. Reduction reaction

PCP dissolved in methanol was reduced to OH-CP using sodium thiosulfate (Fig. 2) in the presence of acetate buffer [12]. OH-CP is relatively unstable and hence has to be generated from PCP. The progress of the reaction was monitored by LC–MS–MS. The precursor–product transitions of 293.1→259.1 and 277.1→142.0 were determined for the $[M+H]^+$ ions for PCP and OH-CP from this solution. These MRM channels were used to estimate the amount of conversion of PCP to OH-CP. The reduction experi-

Table 1
MRM parameters for the analytes during LC–MS–MS

Analyte	Precursor ion (m/z)	Product ion (m/z)	Collision energy (V)	Cone voltage (V)
CP	261.0	140.0	25	35
Oxime	306.1	221.1	20	40
I.S.	286.1	147.0	25	35
PCP	293.1	259.1	20	40
OH-CP	277.0	142.0	25	45

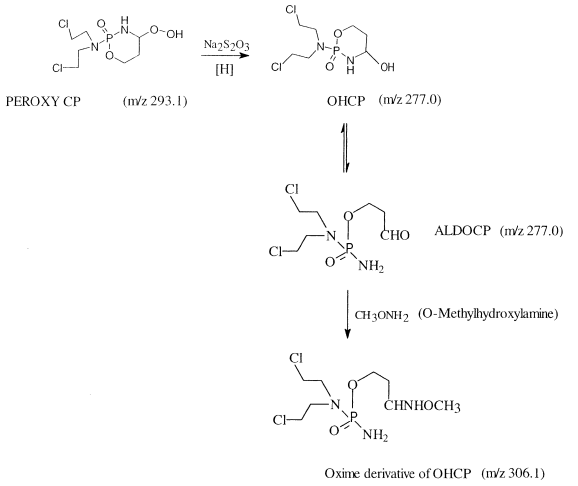

Fig. 2. Reduction and derivatization reactions of PCP and OH-CP.

ment was monitored at two time points $t=0$, and $t=20$ min, which showed that incubation for 20 min resulted in increased signal for OH-CP and decreased signal for PCP. Under these conditions, approximately 50% of PCP were reduced to OH-CP, which was calculated based on the ratio of the peak areas for these analytes at the two time points, and on the assumption that the ionization efficiencies for PCP and OH-CP are identical. The OH-CP formed under these conditions exists in equilibrium with its aldehyde isomer (Fig. 2).

3.2. Oxime derivatization reaction

The derivatization/oxime formation reaction was first optimized using neat solutions. An aliquot of the reduction mixture was reacted with threefold molar excess of the OMHA reagent at 50°C for 20 min. The amine functionality of OMHA reacts with the ALDO-CP isomer to produce the OH-CP oxime (Fig. 2). The oxime derivatization mixture was used to profile the precursor→product transition for the oxime derivative of OH-CP. The ratio of the peak area for OH-CP before and after derivatization was used to estimate the derivatization efficiency. The derivatization experiment was monitored at two time points $t=0$, and $t=20$ min, which showed that incubation for 20 min resulted in increased signal for oxime and decreased signal for OH-CP. The LC–MS–MS response of the oxime ($t=20$ min) was higher than that of OH-CP at $t=0$; hence it can be believed that the ionization efficiency for the oxime was higher than OH-CP. Based on these experimental results (ratio of the peak areas), the derivatization reaction was at least 50% efficient.

These percentage conversions were used in estimating the concentration of the oxime present in the standard plasma samples. However, synthesis of pure oxime reference material would be the best strategy to obtain accurate concentration data. Due to the limited number of study samples and the fact that relative concentrations would be sufficient, the oxime reference standard was not synthesized.

3.3. HPLC method development

A reversed-phase LC method using a YMC basic column was developed for the separation of the different analytes (PCP, OH-CP, oxime, CP and the I.S.). Separation of the mixture was attempted using a mobile phase composed of acetonitrile–0.1% formic acid (50:50), which resulted in the compounds eluting close to the void (~0.7 min). The aqueous content of the mixture was then increased by 10%; a mixture of 0.1% formic acid–acetonitrile (60:40) provided adequate retention for the compounds between 1 and 1.6 min. This is adequate retention for compounds extracted from biological matrices; retention times greater than 1 min on a 50 mm long column help avoid most matrix interference that elute close to the void (void volume elutes at ~0.7 min in a 50×2.0 mm column). The short cycle time (2.5 min) of this method allows high throughput analysis with minimal matrix interference.

The I.S. was chosen based on its structural similarity with the other analytes. Its structure is shown in Fig. 3. Different precursor–product ion transitions for the compounds were chosen so as to prevent any possible cross talk, particularly because similar prod-

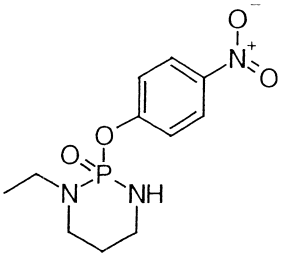

Fig. 3. Internal standard (I.S.) structure.

uct ions were possible for PCP and OH-CP. Fig. 4a and b show the MRM traces for oxime, PCP, I.S., OH-CP and CP at the lowest level on the standard curve obtained for the plasma (3.1 ng/ml of oxime and CP) and spleen (125 ng/g of oxime, 1250 ng/g of CP) matrices, respectively. The chromatographic profiles of the plasma and the tissue extracts were quite similar. The peak areas for the analytes from these chromatograms suggest that it would be possible to quantitate below the limit of quantitation (up to an order of magnitude) set in these experiments in both matrices for the oxime and CP. However, these concentrations are based upon the approximate efficiencies of the reduction and derivatization reactions (~50% each).

3.4. Sample processing

Due its instability, the OH-CP must be trapped immediately upon blood and tissue collection. The trapping/derivatization reaction for plasma study samples was adapted from Baumann et al. [8]. Blood was collected in tubes containing OMHA (15 mg/ml) and the plasma samples were harvested. The samples were heated at 50°C for 20 min to enable complete oxime formation. It was also necessary to derivatize the spleen samples as quickly as possible to ensure sufficient trapping of the OH-CP. Spleen samples were snap frozen immediately after harvesting; OMHA in water was added, followed by homogenization.

3.5. Quantitative analytical parameters

A standard curve analysis for quantitating the oxime involved preparation of the OH-CP from PCP via reduction and further derivatization of the OH-CP using OMHA. For plasma samples, OH-CP oxime standard concentrations were estimated at 3.1, 12.5, 125, 250, 500, 1000, and 3333 ng/ml. For spleen samples, the OH-CP oxime standard concentrations were estimated at 125, 1250, 2500, 5000,

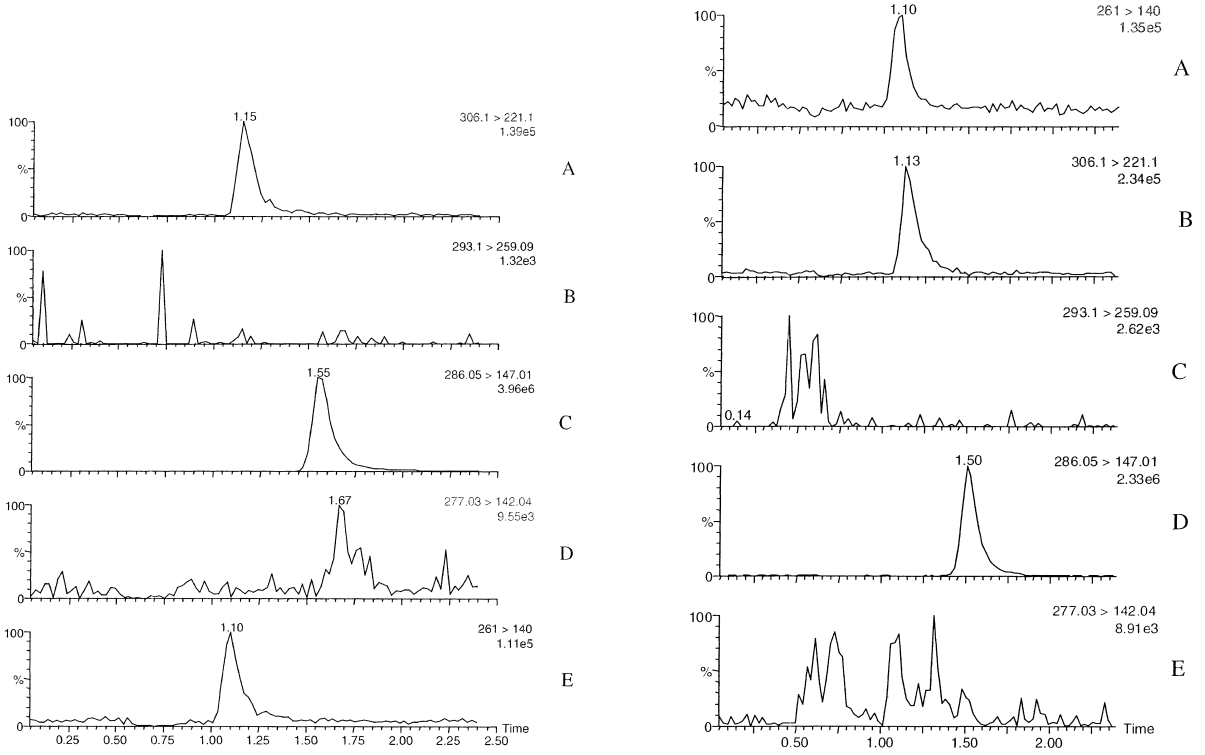

Fig. 4. (a) LC–MS–MS chromatograms for oxime (A), PCP (B), I.S. (C), OH-CP (D) and CP (E) from plasma extracts at 3.1 ng/ml of oxime and CP and 2000 ng/ml of I.S. (b) LC–MS–MS chromatograms for CP (A), oxime (B), PCP (C), I.S. (D), and OH-CP (E) from spleen extracts at 125 ng/g of oxime and 1250 ng/g CP and 20 000 ng/g of I.S.

Table 2
Quantitative performance of the analytical method

Analytical criterion	Plasma		Spleen	
	CP	Oxime	CP	Oxime
Linear range	12.5–3333 ng/ml	3.1–3333 ng/ml	1250–20 000 ng/g	125–50 000 ng/g
Limit of quantitation	3.1 ng/ml	3.1 ng/ml	1250 ng/g	125 ng/g
Slope of correlation plot	0.0018	0.0029	89.87	190.39
Intercept	0.024	0.018	52 086.1	9576.6
R^2	0.99	0.99	0.94	0.99
Accuracy (back calculated standards) (%)	±10%	±15%	±29%	±28%

10 000 and 50 000 ng/g. Calculated concentrations for the study samples were within the concentration limits set by this standard curve. The correlation coefficients for standard curves of both analytes in the two matrices were 0.99 and the accuracy was within ±30%. The inter-day variability was determined to be less than 10%, based on the peak areas of the lowest standard for the oxime, using the same OH-CP stock solution to spike into the matrix followed by derivatization using OMHA. The analytical method parameters for the assay are shown in Table 2. It was not possible to determine the recovery, since a pure reference standard for the oxime did not exist.

3.6. Tissue distribution analysis

This pilot study was performed to demonstrate the utility of this method for the determination of CP and oxime plasma and tissue concentrations. The plasma concentration–time profiles for CP and the oxime following 120 mg/kg, 65 mg/kg, or 40 mg/kg i.v. bolus doses are shown in Fig. 5. The exposures of both CP and oxime increase with CP dose amount, as expected. CP and oxime levels were detectable at 2-h post dose only for the highest dose level, and no detectable concentration of either CP or oxime was observed at the 4-h time point for any dose amount. Fig. 6 shows the CP and oxime concentrations in the spleen following a 120 mg/kg dose. Spleen samples were not collected for the 40 mg/kg or 65 mg/kg dosed mice. The CP levels in the spleen and plasma were similar while the oxime levels in the spleen were significantly lower than the plasma. In experiments not reported, the method used for spleen analysis (described in this article) was successfully used to measure both compounds in tumors.

The ability to measure CP and OH-CP in plasma and tissue will be very useful in future pharmacokinetic and drug interaction studies to determine how metabolism changes affect the plasma and tissue kinetics. It has been previously demonstrated that CP plasma exposure alone does not predict efficacy better than the amount of drug dosed [11]. It has also been shown that increased plasma exposure

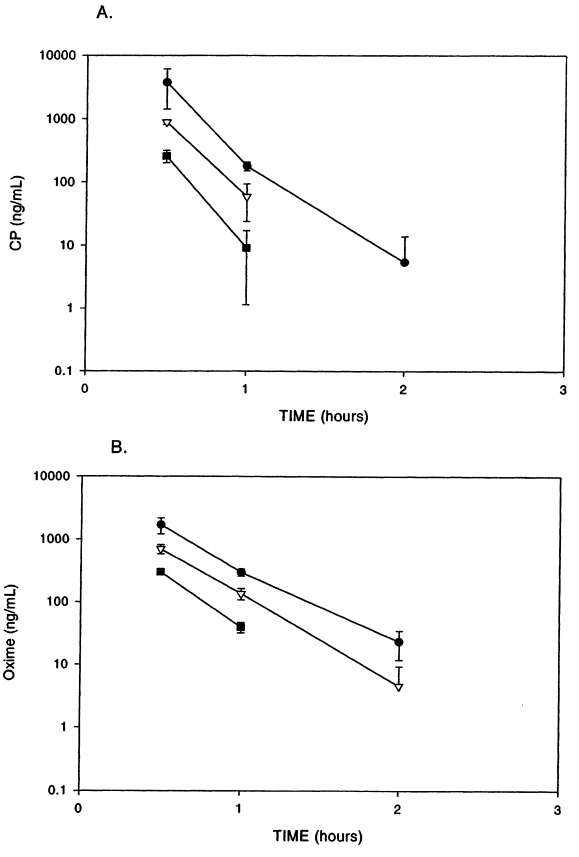

Fig. 5. Mean (±SD) CP (A) and oxime (B) plasma concentration time profiles following 120 mg/kg (●), 65 mg/kg (▽), and 40 mg/kg (■) i.v. bolus doses.

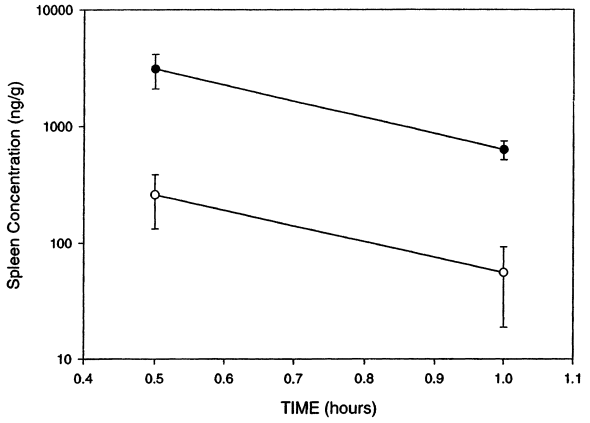

Fig. 6. Mean (±SD) CP (●) and oxime (○) spleen concentration–time profiles following 120 mg/kg i.v. bolus dose.

of activated CP due to metabolic changes does not significantly change the efficacy [13]. Therefore, the ability to monitor changes in concentrations of OH-CP and CP in the tumor, along with the plasma, will allow for the investigation of relationships between tumor and plasma levels that will help in the generation of new models to predict efficacy.

4. Conclusions

Cyclophosphamide and its metabolite, 4-hydroxy-cyclophosphamide, have been quantitated in mouse plasma and tissue by derivatization combined with LC–MS–MS. The derivatization was conducted immediately upon sample collection to trap the OH-CP metabolite intermediate prior to further conversion to phosphoramide mustard or other reaction products. This simple and straightforward derivatization procedure, combined with sample extraction via protein precipitation, allowed quantitation of CP and the oxime derivative of OH-CP in plasma for concentrations ranging from approximately 12.5–3333 ng/ml, and in spleen tissue for concentrations of 1250–50 000 ng/g. The short cycle time (2.5 min) of the LC–MS–MS method allowed high throughput analysis with minimal matrix interference.

Mouse plasma levels were quantitated for doses of 40, 65 and 120 mg/kg; spleen concentrations were determined for mice dosed at 120 mg/kg. The CP and oxime plasma levels correlated well with dose amounts. The CP levels in the spleen and plasma were similar while the oxime levels in the spleen were significantly lower than the plasma. The utility of this analytical method to measure tissue concentrations has been demonstrated, and will be applied in future work to tumor tissue samples, with emphasis on efficacy in tumors and relationships to pharmacokinetics/pharmacodynamics.

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