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New liquid chromatographic assay with electrochemical detection for the measurement of amifostine and WR1065

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Abstract

A high-performance liquid chromatographic method (HPLC) was developed for the analysis of the radio- and chemo-protectant, amifostine and its active metabolite-WR1065 in deproteinized human whole blood and plasma. The two compounds were quantified by measuring WR1065 after two different sample pretreatment procedures. During these procedures, amifostine was quantitatively converted into WR1065, by incubating the sample at 37 °C for 4 h at pH<1.0. The resulting amounts of WR1065 were determined by HPLC with coulometric detection (analytical cell: $E_1 = 200$ mV and $E_2 = 600$ mV; guard cell: $E_G = 650$ mV). The WR1065 standard curve ranged from 0.37 to 50.37 μ*M*. The lower limit of quantitation of WR1065 was 0.25 μ*M*. The within- and between-day precisions were ≤4.3% and ≤6.0% for amifostine, ≤4.4% and ≤3.8% for WR1065, respectively. The within- and between-day accuracy ranged from 95.4 to 97.7% and 95.4 to 97.8% for amifostine, and from 97.1 to 101.7% and 97.2 to 99.7% for WR1065, respectively. This method minimizes WR1065 loss during sample preparation, and allows for rapid analysis of both compounds on one system. Furthermore, the application of a coulometric electrode is more efficient and requires less maintenance than previously published methods for the two compounds.

Keywords: Amifostine; WR1065

1. Introduction

Amifostine ([*S*-2-(3-aminopropylamino)ethylphosphorothioate] WR2721, Ethyol®), an organic thiophosphate was originally developed as radioprotectant, is now approved by the FDA for use as a cytoprotective agent in cancer patients receiving cisplatin and cyclophosphamide chemotherapy. As depicted in Fig. 1, amifostine is converted by alkaline phosphatase to the active metabolite WR1065 [2-(3-aminopropylamino)ethanethiol)], which is further metabolized to inactive disulfides. Amifostine serves as a renal protective agent during cisplatin therapy without hindering the antitumor effects of cisplatin. The basis for this selectivity is preferential formation of WR1065 by alkaline phosphatase found in the vascular walls of normal tissues, but not in tumor tissue. In addition, WR1065 formation occurs at a greater rate in normal tissue because the acidic environment of hypoxic tissues such as

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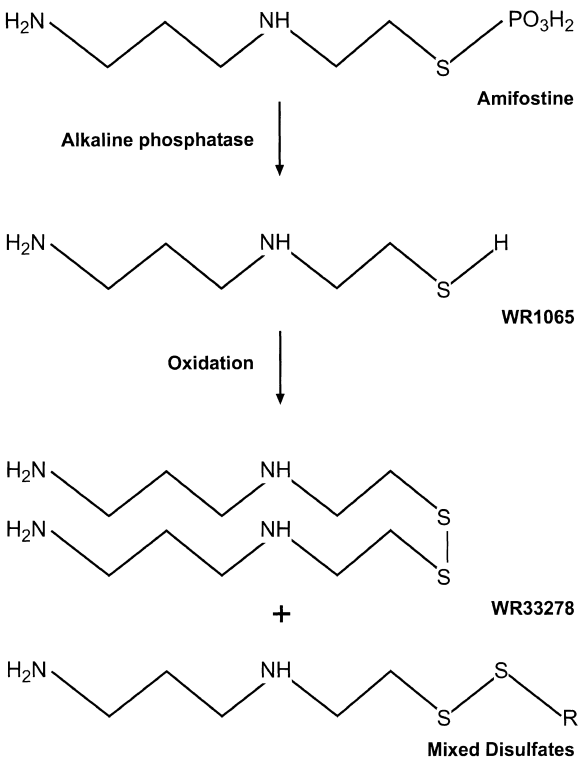

Fig. 1. Metabolic schema for amifostine showing conversion to its active metabolite WR1065 by alkaline phosphatase and then further oxidation to WR33278 and other inactive mixed disulfide metabolites.

tumors also hinders the activity of alkaline phosphatase [1–6].

Most published HPLC assays for amifostine and WR1065 have utilized electrochemical (EC) or fluorescent detection [7–14]. Previously published HPLC–EC assays for amifostine and metabolites in plasma used a traditional amperometric detector such as a Hg/Au electrode. However, the efficiency of traditional amperometry, which measures 5–15% of the electroactive species, is much less than coulometry which measure 100% of the electroactive species. The coulometric detector provides more sensitivity, selectivity, and accuracy than the amperometric detector. Moreover, since amifostine has a short half-life ($<$10 min), and WR1065 is rapidly auto-oxidized in human blood and plasma [8,9], we choose to optimize our ability to measure these compounds with a coulometric detector. Thus in this paper, we describe a rapid and robust reversed-phase HPLC procedure with coulometric detection for the direct measurement of WR1065 in whole blood and plasma, and a sample pretreatment method to prepare whole blood and plasma for indirect measurement of amifostine.

2. Experimental

2.1. Chemicals

Amifostine and WR1065 used for preparation of standards or quality control samples were supplied by US Bioscience (Conshohocken, PA, USA; amifostine: Lot A Sipsy 7, Date 10/00, 79.9% amifostine purity; WR1065: Lot AP-X-61, Date 10/00, 98.7% WR1065 purity). Chloroacetic acid, 99+%, ACS reagent and perchloric acid 70%, redistilled, 99.999% were from Aldrich (Milwaukee WI, USA). HPLC grade methanol was obtained from Burdick and Jackson, Baxter (Muskegon, MI, USA). Triethylamine 99% and EDTA disodium salt, 0.5 *M*, and octyl sulfate sodium salt, $\geq$99% were obtained from Sigma (St. Louis, MO, USA). All water was from a Milli-QUV Plus water system (Ultra-pure water system; 18.2 MΩ).

2.2. Equipment

The HPLC system consisted of an ESA solvent delivery module—Model 582 with Rheodyne 9125 manual injector, an ESA electrochemical detector—Coulochem® II with coulometric electrode (ESA, Chelmsford, MA, USA), a Shimadzu low-pressure gradient flow control valve: FCV-10Alvp with mixer SUS and Shimadzu on-line degasser-DGU-14A (Shimadzu, Kyoto, Japan). A Shimadzu CLASS-VP chromatography data system (version 5.0) was used for data acquisition and processing.

A 15-μl volume of the standard and sample was injected onto a Prodigy 5 μm ODS-3v (150×4.6 mm, Phenomenex, Torrance, CA, USA) with MetaGuard filter (4.6 mm Inertsil ODS-3, 5 μm). Analytes were isocratically eluted with methanol–aqueous mobile phase containing 0.14 *M* chloroacetic acid, 2.2 m*M* octyl sulfate sodium salt and 7.5 m*M* triethylamine (pH 3.0), (37:63, v/v). The flow-

rate was 1.0 ml/min and the column temperature was 40 °C.

WR1065 was detected after HPLC separation with a model Coulochem II EC detector (ESA) equipped with a dual analytical cell (model 5010) and a guard cell (model 5020). The guard cell (E_G) is connected in line before the injector and is used to remove oxidizable impurities in the mobile phase that may interfere with baseline stability. The dual analytical cell contains two porous graphite electrodes in series. The first electrode (E_1) is set at a lower voltage than the second electrode (E_2) and is used as an oxidative screen to eliminate interfering compounds that oxidize at lower potentials than the compounds of interest in the injected sample. For selectivity, E_2 is set at or above the established oxidation potential of the compound of interest. For optimum detection of WR1065, the electrode potentials for the E_G, E_1 and E_2 were set at 650, 200 and 600 mV, respectively. To assure baseline stability before a sample run, the potential at each coulometric electrode was increased stepwise to the final working potential from low to high such as E_2 from 200 to 400, then to 600 mV. The total analysis time of a sample was 10 min.

2.3. Sample preparation

2.3.1. Standard solutions

The amifostine stock solution was made by dissolving 1.0 mg of amifostine in 1.0 ml water. The WR1065 stock solution was made by dissolving 1.0 mg of WR1065 in 1.0 ml 0.2 *M* perchloric acid–0.1 *M* chloroacetic acid, pH 3.0 (3:1, v/v). Aliquots of the stock solutions were stored at −70 °C, and are stable for at least 3 months (i.e. <5% loss). WR1065 calibration standards were prepared at the time of assay from the 1.0 mg/ml stock solution of WR1065 by making dilutions with blank deproteinized blood or deproteinized plasma to obtain calibrators for the standard curve: 0.37, 0.75, 1.49, 2.98, 5.97, 11.9, 23.9 and 50.4 μ*M*. The linear regression of the peak height of WR1065 was weighted by $1/\chi^2$. The squared correlation coefficient was used to evaluate the linearity of the calibration curve.

2.3.2. Quality control

WR1065 quality control samples were prepared at the time of the analysis with blank deproteinized blood or plasma. WR1065 quality control concentrations in whole blood were 3.73, 14.9 and 37.3 μ*M* and in plasma were 4.48 and 29.84 μ*M*. Likewise amifostine quality control concentrations in blood were 9.35, 23.4 and 37.4 μ*M*, and in plasma were 4.67 and 28.04 μ*M*. The amifostine samples were then incubated at 37 °C for 4 h to convert amifostine to WR1065 prior to injection.

2.4. Validation assay

The procedures developed for the quantitation of the two compounds were validated over 4 days in plasma, and 5 days in whole blood by analysis of quality control samples to determine within- and between-day precision and accuracy. Calibration standards were analyzed in duplicate.

The limit of detection (LOD) and limit of quantitation (LOQ) were defined as the ratio of signal-to-noise ≥3 and 10, respectively. These were determined by triplicate analysis of an extensive calibration curve in the low concentration range (e.g. 0.001–0.37 μ*M*).

2.5. Patient sample collection

2.5.1. Whole blood samples

To determine whether this method could reliably detect amifostine and WR1065 whole blood and plasma concentrations in samples collected from patients enrolled on amifostine pharmacokinetic studies, we studied one patient after receiving a 1-min bolus injection of amifostine 600 mg/m^2. Samples were collected serially after the end of the injection and whole blood samples (1.0 ml) were immediately mixed with 1.0 ml ice-cold solution of 0.5 *M* $HClO_4$ and 1.0 m*M* EDTA in a 5 ml purple-top Vacutainer tube (Franklin Lakes, NJ, USA). These tubes were inverted for 15 s before centrifugation at 16 000 *g* and 0 °C for 2 min. The supernatant was transferred directly into a screwcap tube, snap-frozen in liquid nitrogen, and stored at −70 °C until analysis. Drug-free blank deproteinized blood for calibration curves and quality control samples were prepared similarly. As shown in Fig. 2, two different pretreatment methods were used to prepare samples for measurement of amifostine (procedure B) and WR1065 (procedure A).

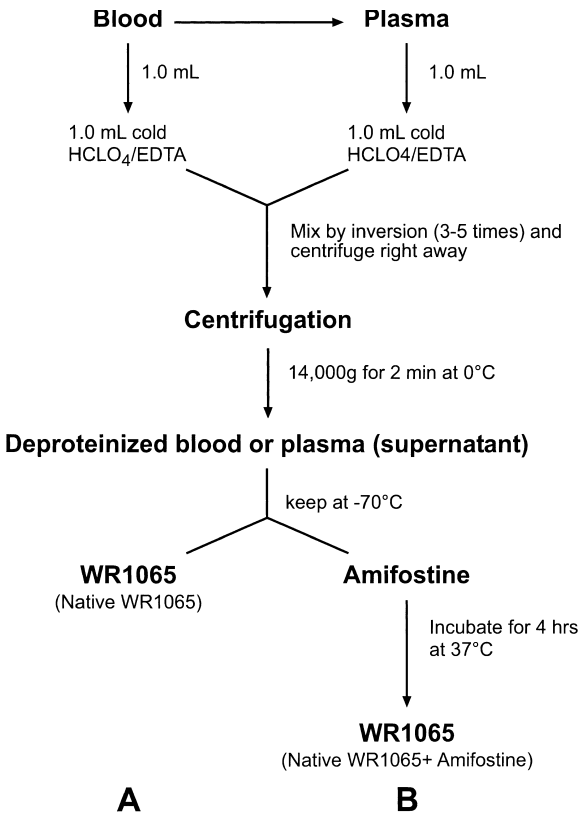

Fig. 2. Sample pretreatment schemes for amifostine and WR1065 measurement in whole blood and plasma.

2.5.2. Plasma samples

Likewise, whole blood samples were collected in 5-ml purple-top Vacutainer tubes and then centrifuged at 16 000 *g* for 2 min to separate the plasma. The resultant plasma (1.0 ml) was prepared similarly to whole blood and analyzed as described previously (Fig. 2).

3. Results

3.1. Chromatography and detection

Since WR1065 is the dephosphorylated free thiol metabolite of amifostine, we used reversed-phase chromatography with a mobile phase at pH 3.0 and sodium octyl sulfate as an ion-pairing reagent for best results. Methanol 37% (test range 20–45%) in mobile phase with a column temperature of 40 °C produced the best resolution from endogenous substances and a favorable retention time. Fig. 3a shows a chromatogram of blank deproteinized blood from a normal volunteer with and without the addition of WR1065. Fig. 3b depicts blank deproteinized plasma with and without WR1065. No interfering peaks were detected at the retention times of WR1065.

To determine the optimal electrical potential for WR1065 at analytical electrode (E_2) (Fig. 4), we tested a series of increasing voltages to find the combination that would give maximal peak height without interfering peaks. For example, the highest voltage we tested (i.e. 800 mV) yielded the best peak height for WR1065; however, we also observed an interfering peak (Fig. 4, insert). Thus, we choose 600

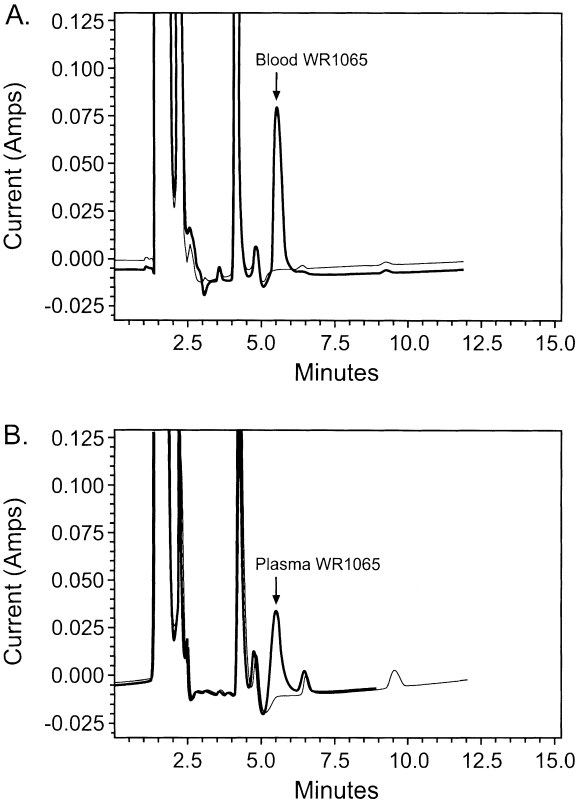

Fig. 3. (a) Representative chromatogram of deproteinized whole blood with and without WR1065; (b) Representative chromatogram of deproteinized plasma with and without WR1065.

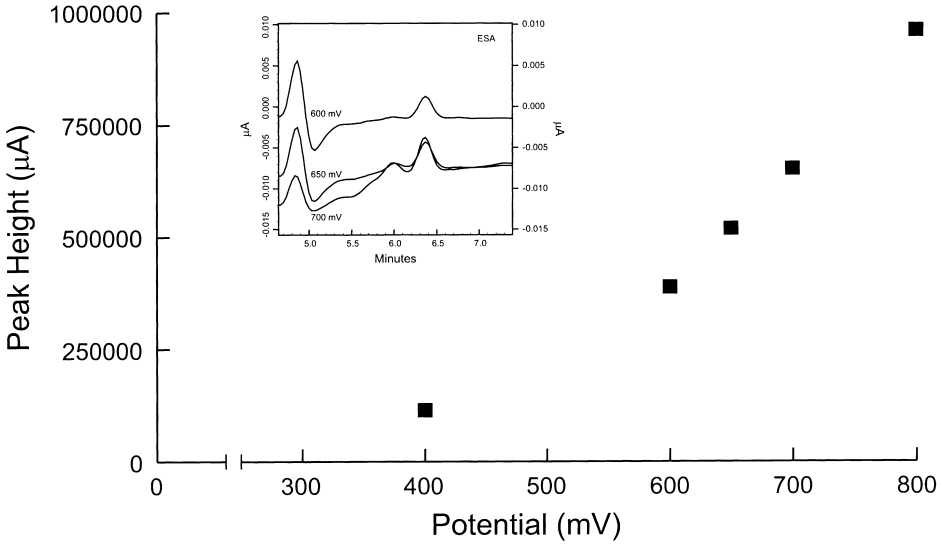

Fig. 4. Plot of WR1065 peak height vs. coulometric electrode potential at E_2 as a measurement of WR1065 maximum electrical potential in deproteinized blood, and depiction of chromatograms at incremental E_2 voltages (inset) depicting presence or absence of interfering peaks in blank deproteinized blood at WR1065 retention time.

mV for E_2 since it gave a suitable peak height for WR1065 without interfering peaks.

3.2. Sample pretreatment method

To establish our sample pretreatment method we varied the perchloric acid concentration as well as the length of sample incubation. In order to optimize human blood and plasma deproteinization in our sample pretreatment method, we tested various concentrations of perchloric acid as shown in Table 1. We evaluated for the presence or absence of a clear supernatant after various concentrations of perchloric acid–EDTA were added to fixed amounts of plasma (1:1, v/v) followed by centrifugation at 16 000 *g* and 0 °C for 2 min. We also measured the WR1065 peak height in these samples to determine if interfering peaks were present. Based upon the results from these studies we choose 0.5 *M* concentration of perchloric acid for our sample pretreatment. Once we established the perchloric acid concentration for our sample pretreatment method, we varied the length of sample incubation to find the shortest time for complete amifostine conversion to WR1065 at 37 °C. Complete conversion of amifostine (11.68 μ*M*) to equimolar WR1065 at 37 °C was observed at 4 h (Fig. 5).

3.3. Assay validation

To assess within- and between-day variability in assay performance, precision, and accuracy, we evaluated validation parameters for WR1065 and

Table 1
Determination of optimal perchloric acid concentration in the deproteinized solution[a] for amifostine and WR1065 human whole blood deproteinization

	Perchloric acid concentration (*M*)			
	0.1	0.5	1.0	3.0
Cloudy supernatant (plasma)	Cloudy	Clear	Clear	Clear
Interference (blank blood)	N/D	No	No	No
Peak height of 1.12 μ*M* whole blood WR1065	N/D	1528	1567	1503

[a] Containing 1.0 m*M* EDTA.

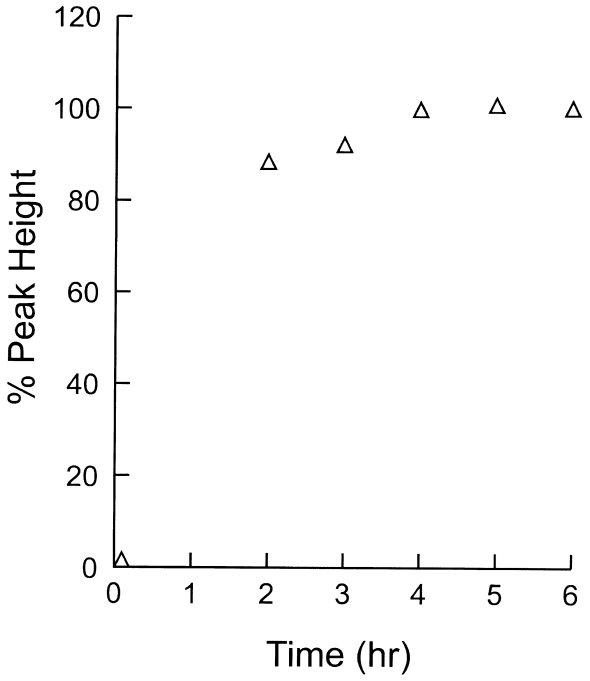

Fig. 5. Plot of percentage WR1065 peak height vs. time as a measure of the complete in vitro conversion of amifostine to WR1065 in whole blood at 37 °C.

amifostine (Table 2). Ten injections of low, medium, and high quality control samples as described previously were made on day 1 to assess within-day variability and again on day 4 or 5 for between-day variability. The LOD and LOQ values of WR1065 were 0.075 μ*M* and 0.25 μ*M* in both deproteinized blood and plasma, respectively. The calibration curves for both blood and plasma were linear up to 50.4 μ*M*.

3.4. Stability

To test for sample stability at −70 °C after processing as described, we measured amifostine and WR1065 concentrations at incremental time points. Fig. 6 depicts the stability of amifostine, 11.7 μ*M* and WR1065, 7.5 μ*M* in deproteinized blood over time at −70 °C. The figure also depicts the stability of amifostine, 29.84 μ*M* and WR1065, 4.48 μ*M* in deproteinized plasma over time at −70 °C. Within 48 h we observed less than a 6.5% decrease in peak height for both amifostine and WR1065 in whole blood samples, and a 9.4% decrease in deproteinized plasma. However, we observed a 20% decrease of WR1065 peak height in whole blood at 72 h, and a

Table 2
Validation parameters of amifostine and WR1065 in deproteinized blood and plasma

Quality control sample (μ*M*)	Within-day ($n=10$)		Between-day ($n=13$)	
	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)
Whole blood				
WR1065				
3.73	4.39	100.1	3.46	99.3
14.9	4.22	101.7	3.79	99.7
37.3	3.34	97.1	3.12	97.2
Amifostine				
9.35	3.16	97.7	5.96	95.4
23.4	3.63	96.1	5.55	95.7
37.4	4.33	95.4	4.66	97.8
Plasma				
WR1065				
4.48	4.96	98.3	5.94	95.5
29.84	2.21	99.2	4.74	96.7
Amifostine				
4.67	4.15	96.9	6.01	95.4
28.04	3.17	98.7	4.82	97.1

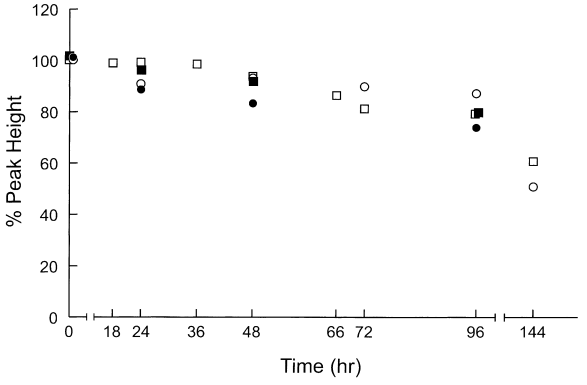

Fig. 6. Plot of % WR1065 peak height vs. time as a measurement of amifostine (○) and WR1065 (□) stability in whole blood, as well as amifostine (●) and WR1065 (■) stability in plasma at −70 °C.

similar decrease in plasma at 96 h. These results led us to conclude that patient samples should be analyzed for WR1065 within 48 h of collection.

3.5. Application of assay in patient samples

Fig. 7 is a blood and plasma concentration versus time profile for amifostine and WR1065 from a patient after treatment with a 1-min bolus injection of 600 mg/m^2 amifostine. All samples were analyzed within 24 h. WR1065 concentrations were higher (1.5–2.5 times) in whole blood than in plasma; however, amifostine concentrations did not differ between whole blood and plasma.

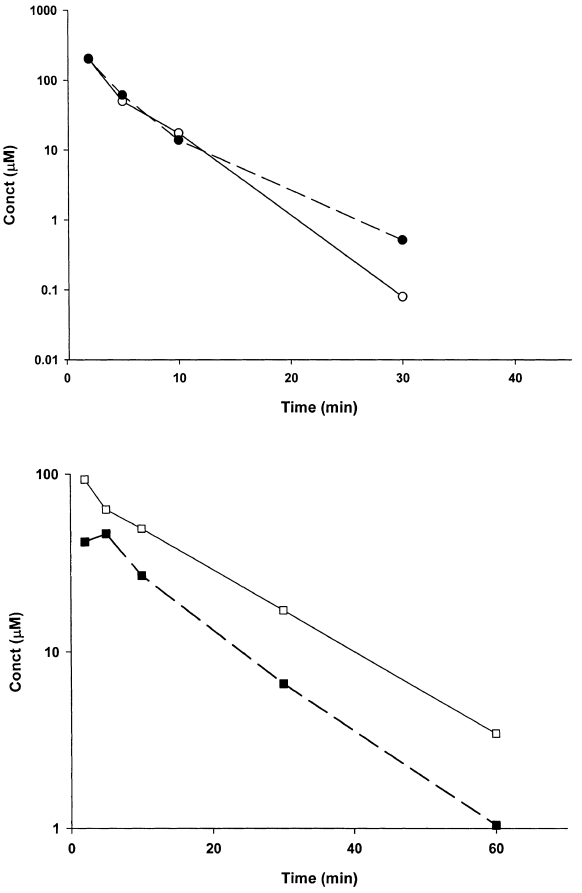

Fig. 7. Concentration–time plots from a patient after treatment with 600 mg/m^2 amifostine depicting amifostine (a) in whole blood (○) and in plasma (●), and WR1065 (b) in whole blood (□), and in plasma (■).

4. Discussion

We report the first whole blood assay in which both amifostine and WR1065 can be measured by one HPLC coulometric detection method. Due to the reported short half-life of amifostine as well as the tendency for WR1065 to undergo immediate oxidation, our desire was to develop a rapid approach to process patient blood samples at the bedside. Once processed, these samples were assayed immediately, which will allow for accurate measurements of both amifostine and WR1065. This accuracy is reflected in our validation results, in which within- and between-day measurements did not exceed 5 and 6% for WR1065 and amifostine, respectively.

Patient pharmacokinetic data for amifostine and its metabolites in plasma have been published previously by Shaw and van der Vijgh and co-workers [11,15–17]. However, we reason that a longer preparation time for plasma versus whole blood may lead to increased WR1065 oxidation. In our own in vitro experiment in which we added WR1065 to plasma, we observed rapid and variable degradation within 3 min of incubation [data not published]. Published methods for WR1065 also report that this agent

undergoes very rapid oxidation while in plasma and blood [7–9]. Taken together, this propensity for rapid WR1065 oxidation led us to develop a procedure that minimizes degradation during sample preparation. We are unaware of other methods that account for this aspect, in combination with a sensitive, accurate, stable, and rapid HPLC–EC method, suitable for studying the disposition of amifostine and WR1065 in human blood. The observation that amifostine concentrations were similar in both blood and plasma may be explained by immediate immersion of both sample sets into ice cold conditions which would tend to inhibit alkaline phosphatase activity and thus slow down conversion of amifostine to WR1065. Shaw et al. incubated amifostine in whole blood and then treated with perchloric acid–EDTA at 0 °C, and they determined that amifostine undergoes hydrolysis under these conditions at 0.6% per hour [8]. In contrast, we observed higher WR1065 concentrations in blood than in plasma. This latter observation is likely due to the early treatment of whole blood with perchloric acid, resulting in a slow down of the oxidative process of WR1065.

Several other factors improved our assay over other previously published work. A coloumetric dual carbon electrode was utilized to measure WR1065 instead of the amperometric electrode such as Hg/Au described previously [7–9]. We chose this electrode because the coulometric detector is more sensitive, selective, and accurate as well as having a smaller requirement for extensive maintenance. For additional optimization, we added octyl sulfate into our aqueous mobile phase with 37% methanol and kept the column temperature at 40 °C to stabilize the retention time of WR1065. This allowed for early WR1065 elution without interfering peaks, and also allowed us to shorten our run time to less than 10 min. A small sample volume injection and addition of TEA into the mobile phase were used to maintain optimal peak shape. We also minimized the perchloric acid concentration during the whole blood and plasma pretreatment in order to maximize the lifetime of our analytical column and to maintain HPLC baseline stability more easily. Finally, direct treatment of blood not only reduced quantitative losses of WR1065, but also simplified our preparation, and thus minimized the necessity for an internal standard in our HPLC method.

In conclusion, we have described a method for the measurement of amifostine and WR1065 in whole blood. This method includes sample preparation, which minimizes loss of WR1065 during the sample preparation, and would allow for more accurate measurement than in plasma. The RP-HPLC uses coulometric electrodes and is a rapid and robust method to measure WR1065 in human blood and an indirect method to measure amifostine, both with one HPLC–EC system.

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