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Determination of WR-1065 in human blood by high-performance liquid chromatography following fluorescent derivatization by a maleimide reagent ThioGlo™3

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Abstract

In order to improve the sensitivity and stability of human blood samples containing WR-1065 (i.e., active metabolite of the cytoprotective agent amifostine), a high-performance liquid chromatographic method was developed and validated using fluorescent derivatization with ThioGlo™3. Using a sample volume of only 100 μl, the method was specific, sensitive (limit of quantitation = 10 nM in deproteinized blood or 20 nM in whole blood), accurate (error ≤ 3.2%) and reproducible (CV ≤ 8.7%). In addition, the stability of WR-1065 in deproteinized and derivatized blood samples was assured for at least four weeks at −20 °C. This method should be particularly valuable in translating the kinetic–dynamic relationship of WR-1065 in preclinical models to that in cancer patients.

Keywords: Blood samples; Fluorescent derivatization; HPLC; ThioGlo™3; WR-1065

1. Introduction

Amifostine [$H_2N(CH_2)_3NH(CH_2)_2SPO_3H_2$; MW = 214] (Fig. 1) is a cytoprotective agent that is currently used to reduce the renal toxicity of cisplatin in patients with advanced ovarian cancer or non-small cell lung cancer, and to reduce the incidence of moderate to severe xerostomia in patients undergoing radiation treatment for head and neck cancer [1]. Being a prodrug, amifostine is effective only after it has been dephosphorylated by alkaline phosphatase in tissues to its pharmacologically active metabolite, WR-1065 [$H_2N(CH_2)_3NH(CH_2)_2SH$; MW = 134]. Protection of normal cells by WR-1065 is usually attributed to its ability to scavenge free radicals, to donate hydrogen ions to free radicals, to deplete oxygen, and to directly bind to reactive metabolites of antineoplastic agents [2,3]. Exposure of cells to WR-1065 can also result in the catalytic inactivation of DNA topoisomerase II, thereby slowing cell cycling and providing more time for DNA repair to occur [4]. The ability of amifostine to preferentially protect normal tissues is due to the higher activity of capillary alkaline phosphatase, and the higher pH and better vascularity of normal tissues relative to that in tumors, thereby resulting in greater cellular levels of the active metabolite [2,3].

To better understand the pharmacokinetic–pharmacodynamic relationship of WR-1065, and for translating preclinical findings to the clinic [5–7], it is important to have an assay method that is accurate, precise and sensitive. For free thiols, especially, it is necessary that biological specimens are collected and processed in a manner that assures drug stability. At present, WR-1065 is typically analyzed using high-performance liquid chromatography (HPLC), coupled to electrochemical or fluorescent detection [8–13]. Unfortunately, some of these assay methods require large

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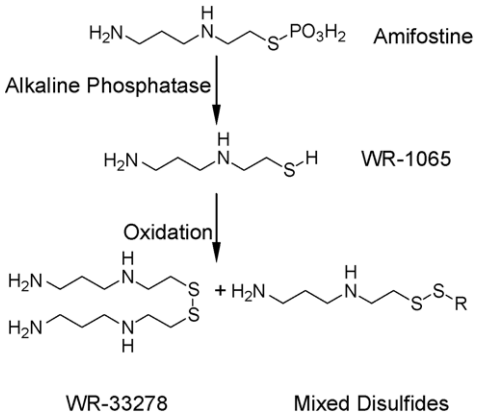

Fig. 1. Metabolic scheme of amifostine in vivo. Amifostine is dephosphorylated by alkaline phosphatase, a plasma membrane bound enzyme, to form the active metabolite WR-1065. The free thiol is further oxidized to form a symmetrical disulfide (i.e., WR-33278) and mixed disulfides, where R represents protein, glutathione or cysteine.

sample volumes while others have either poor sensitivity and/or questionable stability of the analyte in plasma or blood. In particular, a recent assay [13] has strongly articulated that patient samples be analyzed for WR-1065 within 48 h of collection, a requirement that is not always possible or practical. WR-1065 is very unstable in biological specimens because it rapidly forms inactive disulfides (Fig. 1). Moreover, WR-1065 is a small, hydrophilic compound with properties that are similar to that of endogenous free thiols. Thus, it is a challenge to specifically and sensitively measure WR-1065 in biological matrices.

In this paper, we developed and validated an HPLC method for WR-1065 in human blood samples, where the free thiol is coupled to ThioGlo^{TM}3 and then measured by fluorescence detection. Using a 100 μl aliquot of sample, the method was specific, sensitive (limit of quantitation = 10 nM in deproteinized blood or 20 nM in whole blood), accurate (error ≤ 3.2%) and reproducible (CV ≤ 8.7%). Moreover, the method was simple to perform and assured the stability of WR-1065 at −20 °C for at least four weeks.

2. Experimental

2.1. Chemical and reagents

Amifostine and WR-1065 standards were generously provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute (Bethesda, Maryland). 9-Acetoxy-2-(4-(2,5-dihydro-2,5-dioxo-1H-pyrrol-1-yl)phenyl)-3-oxo-3H-naphtho[2,1-b]pyran (i.e., ThioGlo^{TM}3) was purchased from the Calbiochem–Novabiochem Corporation (San Diego, CA). Perchloric acid 70% (redistilled) was obtained from the Aldrich Company (Milwaukee, WI). EDTA disodium salt and potassium phosphate monobasic and dibasic salts were purchased from the Sigma Chemical Company (St. Louis, MO). HPLC grade acetonitrile, water, phosphoric acid and glacial acetic acid were purchased from Fisher Scientific (Pittsburgh, PA). Other chemicals were obtained from standard sources and were of the highest quality available.

2.2. Sample processing

Aliquots of blood were immediately placed in prechilled tubes containing an equivalent volume of 0.5 M perchloric acid and 2.7 mM EDTA solution in order to stabilize WR-1065. Samples were mixed together thoroughly and then centrifuged at 13,000 × *g* for 8 m at 0 °C. The supernatant (i.e., deproteinized blood) was collected and stored at −80 °C until derivatization with ThioGlo^{TM}3. Sample derivatization was performed within 8 h of sample collection.

2.3. Sample derivatization

WR-1065 was derivatized with the maleimide reagent, ThioGlo^{TM}3, in order to form a fluorescent adduct (Fig. 2). A 100 μl aliquot of deproteinized blood was added to a solution containing 95 μl of 100 mM potassium phosphate buffer (pH 7.2), 55 μl of 0.5 M NaOH and 700 μl acetoni-

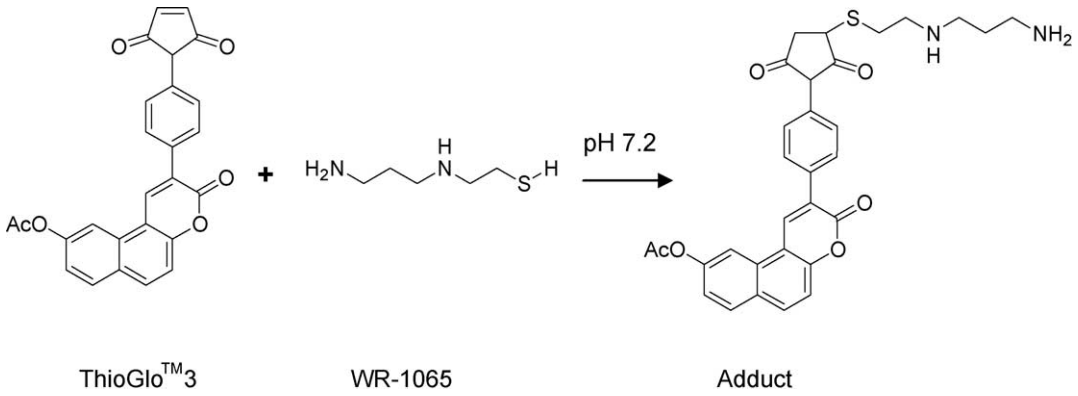

Fig. 2. Schematic for the formation of the fluorescent adduct, WR-1065–ThioGlo^{TM}3.

trile, resulting in a pH of about 7.0 for the mixture. A 50 μl aliquot of 1 mg/ml ThioGlo^{TM}3 solution in acetonitrile was then added and mixed to give a 1 ml final incubation volume, followed by a 10 min incubation period at room temperature. Incubation was stopped by the addition of 10 μl of 2N HCl to the reaction mixture. This solution had a pH of about 1.8 so the WR-1065 derivative was stable. The sample was filtered through a nylon membrane (0.2 μm pore size; Millex®-GN, Fisher Scientific, Pittsburgh, PA) and a 50 μl injection volume was then introduced into the HPLC system.

2.4. Instrumentation

WR-1065 concentrations in blood were determined by an HPLC system consisting of a 600S controller, a 616 pump and a 717 plus autosampler (Waters Corporation, Milford, MA), coupled to an RF-551 spectrofluorometric detector (Shimadzu, Columbia, MD) and a 900 Series Interface running Turbochrome (6.1) software (Perkin-Elmer Nelson, San Jose, CA) for data acquisition and processing. Excitation and emission wavelengths of 365 and 445 nm, respectively, were selected for fluorescent detection. HPLC separations were carried out on a Phenomenex® (Torrance, CA) Synergi 4 μ hydro-RP C18 column (150 × 4.6 mm) at a flow rate of 1.2 ml/min. The mobile phase consisted of 0.1% phosphoric acid plus 0.1% acetic acid in either water (A) or acetonitrile (B), with a gradient elution running at the following: 0–7 min (28–35% B); 8–18 min (50–70% B); and 19–24 min (28% B) (Fig. 3). Peak areas of the WR-1065 adduct in patient samples were compared to standard curves constructed over the 10–2000 nM concentration range in deproteinized blood (corresponds to 20–4000 nM in whole blood) for the determination of drug levels.

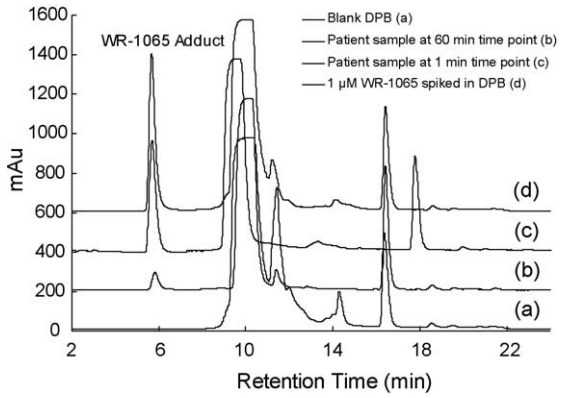

Fig. 3. Chromatograms were obtained from: (a) blank blood; (b) patient blood sample containing WR-1065 collected 60 min after dosing amifostine; (c) patient blood sample containing WR-1065 collected 1 min after dosing amifostine (sample was diluted 20-fold with 0.175 M perchloric acid plus 0.945 mM EDTA solution); and (d) deproteinized blood spiked with 1 μM of WR-1065. Retention time of the WR-1065 adduct is 5.6 min. DPB is deproteinized blood.

2.5. Calibration standards and quality controls

Calibration standards for WR-1065 were prepared in water, medium (i.e., 0.175 M perchloric acid plus 0.945 mM EDTA solution) or deproteinized blood at concentrations of 10, 25, 50, 100, 500, 1000 and 2000 nM. Quality control samples for WR-1065 were prepared in water, medium or deproteinized blood at concentrations of 10, 100 and 1000 nM. Calibration standards and quality control samples were prepared at the time of assay. However, stock solutions (i.e., medium) can be stored at −80 °C for at least one week, a finding that is consistent with other investigators [11,13]. The derivatization of WR-1065 in medium and deproteinized blood was performed as described previously. A minor modification, however, was made for the derivatization of WR-1065 in water, in which 100 μl of the standard was mixed with 150 μl of water instead of 0.5 M NaOH and 100 mM potassium phosphate buffer. The remaining steps were as described before. Calibration data were best fitted to a power function described by the equation: $\ln(Y) = \text{slope} \cdot \ln(X) + y\text{-intercept}$, where ($Y$) is the peak area and ($X$) is the concentration of WR-1065. Although a ln–ln transformation of the data was performed, the relationship between peak area and WR-1065 concentration was linear since the slope of the line was essentially equal to one. Regression parameters (i.e., slope and y-intercept) were then used to calculate the concentration of WR-1065 in quality control samples and in vivo blood samples.

2.6. Patient study

In order to validate the assay procedure, a patient with liver cancer was studied after receiving an intravenous bolus injection of amifostine (Ethyol®) in one arm at a dose of 340 mg/m^2. Serial blood samples were collected from a peripheral vein in the other arm just prior to dosing, at 1, 5, 15, 30, 45 and 60 min, and at 2, 3, 4, 5 and 6 h after dosing. The research protocol followed the tenets of the Declaration of Helsinki promulgated in 1964 and was approved by the institutional review board of the University of Michigan. Informed consent was obtained.

3. Results

3.1. Chromatography

Representative chromatograms of WR-1065 in deproteinized blood (with and without spiked compound) and patient samples are shown in Fig. 3. No chromatographic peaks of the WR-1065 adduct (retention time of 5.6 min) were observed in blank deproteinized blood, indicating that endogenous compounds do not interfere with the method. This lack of interference was confirmed from three different sources of blank human blood. Spiked and patient samples clearly show a clean separation of WR-1065 from the endogenous peaks that elute about 8–20 min after the injection. For those blood

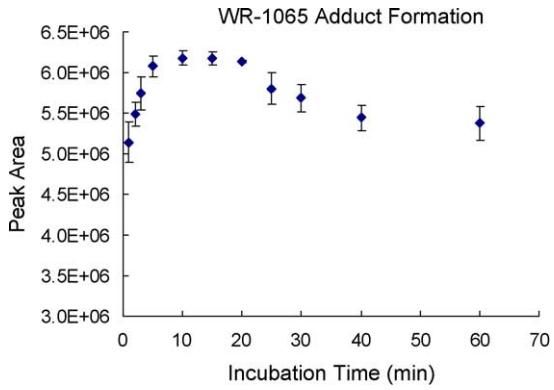

Fig. 4. Formation of the WR-1065 adduct as a function of incubation time. Deproteinized blood was spiked with 1 μM of WR-1065 and reacted with ThioGlo^{TM}3 at room temperature for 60 min. Peak areas are reported as mean values ± standard deviations (SD) of three determinations.

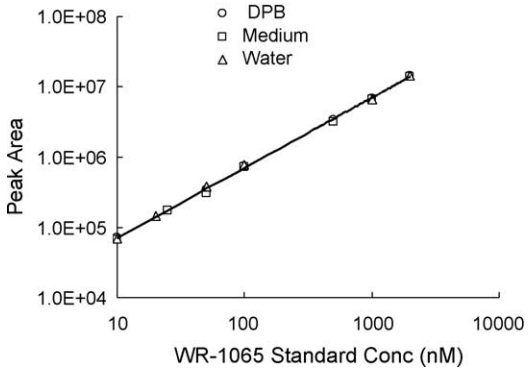

Fig. 5. Representative standard curves obtained for WR-1065 in deproteinized blood (DPB), medium (0.175 M perchloric acid plus 0.945 EDTA) and water. Calibration data were best fitted to a power function described by the following equations: $\ln(Y) = 0.9991 \cdot \ln(X) + 8.8612$ ($R^2 = 0.9996$) for DPB; $\ln(Y) = 0.9792 \cdot \ln(X) + 8.9468$ ($R^2 = 0.9997$) for medium; $\ln(Y) = 0.9841 \cdot \ln(X) + 8.9664$ ($R^2 = 0.9997$) for water. R^2 is coefficient of determination.

samples that were above the linear concentration range of quantitation (usually after the early collection times), further dilution was achieved using medium (i.e., 0.175 M perchloric acid plus 0.945 mM EDTA solution).

3.2. Derivatization

Formation of the WR-1065–ThioGlo^{TM}3 adduct was evaluated as a function of incubation time. As shown in Fig. 4, a 1 μM standard of WR-1065 in deproteinized blood was allowed to react with ThioGlo^{TM}3 for 1, 2, 3, 5, 10, 15, 20, 25, 30, 40 and 60 min at room temperature. The results indicated that the adduct was formed maximally during 5–20 min of incubation, and then slowly decreased during 25–60 min of incubation. The WR-1065 adduct was not stable during longer incubation times. As a result, the 10 min incubation time was selected for assay development and validation of the method.

3.3. Assay validation

Standard curves of WR-1065 were linear over the 10–2000 nM concentration range when prepared in deproteinized blood, medium (i.e., 0.175 M perchloric acid plus 0.945 mM EDTA solution) or water, as shown by the representative examples in Fig. 5. Since the actual blood samples were diluted 2-fold during the initial processing step, this corresponds to a linear concentration range of 20–4000 nM in whole blood ($r^2 \geq 0.999$). The three curves were almost superimposable regardless of the media used, indicating that derivatization of WR-1065 in deproteinized blood or medium was an efficient process as compared to water (i.e., 100.8 ± 1.2% or 97.0 ± 1.9%, respectively; $n = 5$). Table 1 contains the mean results of WR-1065 quality control samples in deproteinized blood at three different concentrations (i.e., low, medium and high). Five replicates were evaluated

Table 1
Assay validation of WR-1065 in deproteinized human blood[a]

Quality control sample	Measured conc. (nM)			
Spiked conc. (nM)	Within-day assay ($n = 5$)		Between-day assay ($n = 5$)	
10.0	Mean	10.0	Mean	10.1
	SD	0.6	SD	0.9
	%CV	5.4	%CV	8.7
	%Error	0.4	%Error	3.2
100.0	Mean	101.8	Mean	101.7
	SD	5.1	SD	6.5
	%CV	5.0	%CV	6.0
	%Error	1.8	%Error	2.8
1000.0	Mean	1002.5	Mean	1021.0
	SD	49.5	SD	39.8
	%CV	4.9	%CV	3.9
	%Error	0.25	%Error	2.1

[a] Precision of the assay method is represented by percentage coefficient of variation (%CV). Accuracy of the assay method is represented by %error, calculated as: 100 × (measured concentration − spiked concentration)/spiked concentration. The within-day assay evaluated 5 replicates ($n = 5$), whereas the between-day assay evaluated 1 replicate per day × 5 days ($n = 5$).

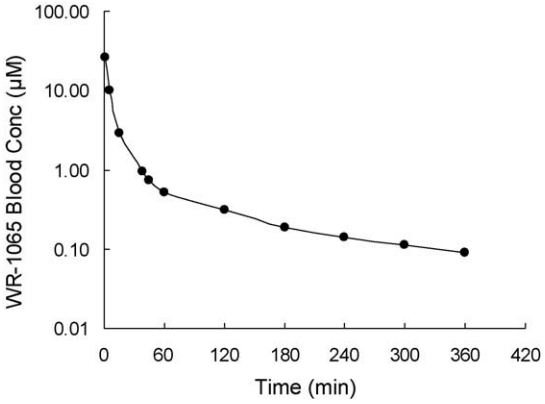

Fig. 6. Concentration–time profile of WR-1065 in blood following an intravenous injection of 340 mg/m^2 amifostine to a patient with liver cancer.

within a day as well as one replicate between several days and, as shown in this table, precision and accuracy of the method were excellent for the 10, 100 and 1000 nM concentrations studied. In all cases, CV was $\leq$ 8.7% and error was $\leq$ 3.2%, regardless of whether the determinations were made intraday or interday. The limit of quantitation for WR-1065 was 10 nM in deproteinized blood, with a signal to noise ratio of 8:1.

3.4. Application of assay in patient blood samples

The blood concentration–time profile of WR-1065 in a liver cancer patient is displayed in Fig. 6. All samples were deproteinized immediately and then analyzed (i.e., derivatized and assayed) within 24 h of collection. Amifostine was rapidly converted to WR-1065 with a peak concentration of 25.8 μM occurring at the first collection time of 1 min. The blood levels of WR-1065 decreased steadily over time in a multi-phasic manner, similar to that observed by others [14], with a log–linear terminal half-life of about 3.2 h.

3.5. Stability

Stability of the patient samples was assessed by comparing the blood concentrations of WR-1065 under different storage conditions. As demonstrated in Fig. 7A, virtually identical curves were obtained for WR-1065 when derivatized blood samples were stored over four weeks at −20 °C. Although a more limited analysis, the derivatized blood samples were also stable over two weeks when stored at 4 °C (Fig. 7B). In contrast, WR-1065 was not stable in deproteinized blood samples (i.e., prior to derivatization) even when stored at −80 °C (Fig. 7C). In this context, WR-1065 degraded to 55–71% of control values at two weeks and to 29–36% of control values at three weeks of storage. Based on this data, the first-order degradation rate constant of WR-1065 in deproteinized samples (at −80 °C) was estimated at 0.05 day^{-1} (half-life of about 14 days).

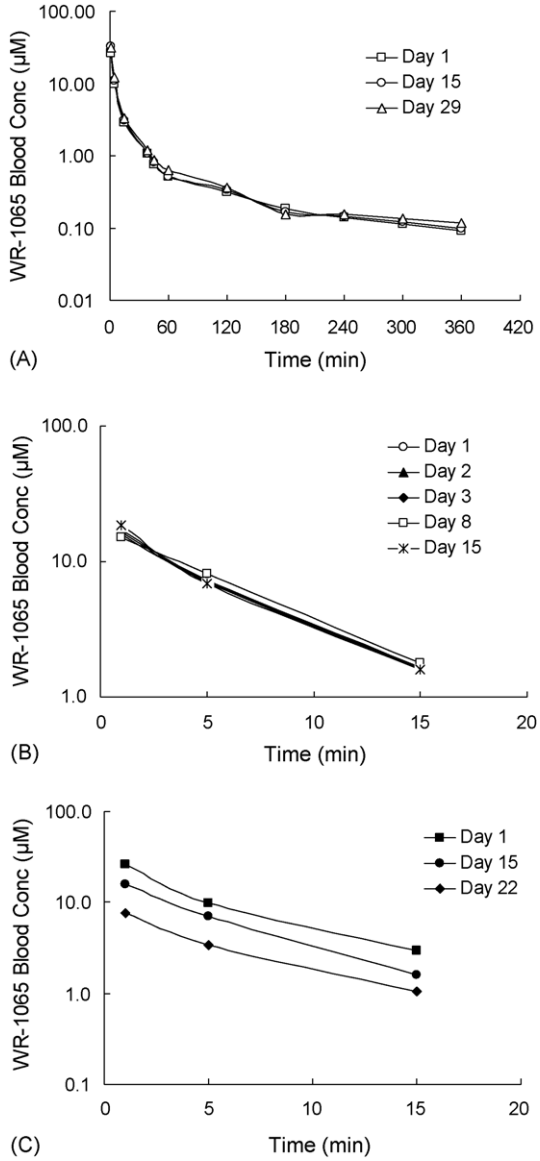

Fig. 7. Stability of WR-1065 in blood samples from a liver cancer patient when stored as: (A) derivatized blood at −20 °C (samples tested at 1 min to 6 h after dosing amifostine); (B) derivatized blood at 4 °C (samples tested at 1, 5 and 15 min after dosing amifostine); and (C) deproteinized (but not derivatized) blood stored at −80 °C (samples tested at 1, 5 and 15 min after dosing amifostine).

4. Discussion

Most published HPLC assays for WR-1065 have been based on electrochemical [8,9,11,13] or fluorimetric detection [10,12]. While each method has merit, there are also some disadvantages associated with the assays. A significant drawback of most the described procedures is the low sensitivity in which the limit of quantitation for WR-1065 ranges from 0.10 to 0.37 μM [8,9,11–13]. In addition, some methods use larger volumes of blood or plasma (e.g., 1 ml) for the analyses [9,11,13]. Although the assay by Mank et al.

[10] has good sensitivity in plasma (i.e., 50 nM), the method employs pre-column derivatization and diode laser induced fluorescence detection, which is costly. Moreover, the method was never validated with in vivo human or preclinical samples (i.e., only spiked plasma was tested). Most importantly, Bai et al. [13] recognized that significant degradation of WR-1065 occurs during sample preparation and storage (i.e., rapid oxidation while in plasma or blood), even at temperatures as low as −70 °C. As a result, these investigators stated that patient samples should be analyzed for WR-1065 within 48 h of collection, an argument that has been generally underappreciated. Notwithstanding this recommendation, the ability to analyze biological samples in such a short period of time is not always possible or practical to accomplish.

Given the need to develop a more sensitive and stable assay method for WR-1065, based primarily on our previous results from preclinical studies [5–7] and preliminary data in liver cancer patients at low amifostine doses (i.e., 340 mg/m^2), a new HPLC/fluorimetric assay was developed. ThioGlo^{TM}3 was used as a derivatizing agent because this reagent has several advantages such as a low fluorescence background, a high quantum yield after reacting with free thiols, a fast reaction time, and a slow hydrolysis rate of the adduct [15,16]. Furthermore, ThioGlo^{TM}3 has been used as a fluorescent probe, in combination with HPLC, to successfully analyze a number of free thiol substrates and drugs in biological samples such as glutathione [17], captopril [16] and MESNA [18].

Using ThioGlo^{TM}3 as a derivatizing agent, the assay sensitivity of WR-1065 in our method (10 nM in deproteinized blood or 20 nM in whole blood) was lower than that reported by previous investigators [8–13]. Moreover, the assay method was relatively simple to perform, accurate as judged by error values of ≤ 3.2% and reproducible as judged by CV values of ≤ 8.7% for both intraday and interday analyses. Of critical importance was the fact that once treated (i.e., the blood deproteinized and an WR-1065 adduct formed), the processed samples could be stored frozen at −20 °C for a minimum of four weeks with no loss of activity. Thus, if processed and stored properly, patient samples yielded the same information whether assayed on day 1 or day 29 after collecting the blood.

Several caveats of the assay procedure are worth mentioning. First, blood samples should be placed on ice and immediately mixed with a degassed, ice-cold solution of 0.5 M perchloric acid plus 2.7 mM EDTA. At a pH of around 0.92, WR-1065 is stable in the deproteinized blood for 24 h at −80 °C. While a deproteinizing solution of 1.0 M perchloric acid plus 2.7 mM EDTA could have also been used to obtain a clear supernatant following centrifugation, the lower concentration of perchloric acid was more amenable to pH control for the subsequent derivatization process. Second, the derivatization of WR-1065 with ThioGlo^{TM}3 should proceed at neutral pH (7.0–7.4) in order to facilitate the reaction. This is accomplished by adding 0.5 M NaOH plus potassium phosphate buffer (pH 7.2) to the deproteinized blood, thereby maintaining the pH at around 7.0. Finally, after incubation at room temperature for 10 min, the samples are acidified with 2N HCl to stop the reaction. The final pH of the solution is around 1.8, which is important for stabilizing the derivatives. Taken as a whole, it appears that pH control is critical, although for different purposes, in the processing and derivatization of blood samples.

It has been reported that amifostine undergoes temperature- and pH-dependent conversion to WR-1065 in deproteinized blood [9,13]. However, the possibility of this occurring during sample derivatization is highly unlikely because the rate of hydrolysis decreases with increasing pH. Thus, at neutral pH, no detectable hydrolysis occurs over 4 h at room temperature [19]. Nevertheless, amifostine (10 μM) was spiked into deproteinized blood (run in triplicate) and carried through the derivatization process. We found that only 0.5% of amifostine appeared as the free thiol WR-1065, demonstrating that WR-1065 is not artificially produced from amifostine.

In conclusion, we have described and validated a new HPLC-fluorescent derivatization method for the measurement of WR-1065 in human blood samples. The method is specific, accurate, reproducible, and more sensitive than previously reported assays. Once processed (i.e., deproteinized and derivatized), the blood samples are stable for at least 14 days at 4 °C and for at least 28 days at −20 °C. This assay is currently being used to evaluate the extent of liver protection afforded by systemic or regional administration of amifostine in preclinical models as well as in the clinic.

Acknowledgments

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