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Short communication

Measurement of both protein-bound and total *S*-2-(3-aminopropylamino)ethanethiol (WR-1065) in blood by high-performance liquid chromatography

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Abstract

A high-performance liquid chromatographic method for automated analysis of both protein-bound and total *S*-2-(3-aminopropylamino)ethanethiol (WR-1065) in blood has been developed in our laboratory. WR-1065 is the active thiol metabolite of the radio- and chemo-protector drug amifostine (WR-2721). Using WR-1065 quality control levels over the experimental range: 7.0, 45.0 and 85.0 μmol/l spiked into plasma, method validation for total WR-1065 included between-run assessment of imprecision (SD/C.V.%: 1.11/16.7%, 6.58/15.5% and 9.24/11.3%, respectively) and % accuracy (94.7, 106.0 and 97.2%). © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

S-2-(3-Aminopropylamino)ethylphosphorothioate (WR-2721, amifostine) is a radio- and chemo-protector drug which is currently used to provide protection to cancer patients undergoing chemo- or radiation therapy. Catalytic hydrolysis of amifostine by alkaline phosphatase takes place at the surface of plasma membrane sites to produce the sulfhydryl metabolite *S*-2-(3-aminopropylamino)ethanethiol (WR-1065), which is considered to be the active form of amifostine.

$H_2NCH_2CH_2CH_2NHCH_2CH_2SPO_3H_2 + H_2O$

S-2-(3-aminopropylamino)ethylphosphorothioate → (WR-2721)

$H_2NCH_2CH_2CH_2NHCH_2CH_2SH + H_3PO_4$

S-2-(3-aminopropylamino)ethanethiol (WR-1065)

Assays for the measurement in blood of the parent drug as well as soluble low molecular weight metabolites have been described previously [1–3]. Amifostine does not bind to plasma proteins [4]. However, at least 50% of WR-1065 is bound to proteins in plasma in the mouse [5], so that measurement of the ''free'' fraction alone does not give a complete picture of the PK of this metabolite.

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Initially, by measuring protein-bound WR-1065 in perchloric acid-precipitated whole blood pellets, formed by treatment of whole blood specimens to produce ''free'' WR-1065 in the supernatant fraction, we were able to show that a significant percentage of WR-1065 binds to plasma proteins. However, the extraction procedure proved complex, and led us to develop the more practical approach of directly measuring total WR-1065 in plasma. Total WR-1065 includes all metabolic forms of this thiol compound: the free thiol form, protein-bound and low molecular weight mixed disulfides and symmetrical disulfide, WR-33278 ($[H_2NCH_2CH_2CH_2NHCH_2CH_2S-]_2$). WR-251833, 3-(4-aminopropylamino)propanethiol ($H_2NCH_2CH_2CH_2CH_2NHCH_2CH_2CH_2SH$), the analogue of WR-1065, was used as an internal standard. Both methods involved initial reduction of protein-bound WR-1065 disulfides to the corresponding thiols, followed by treatment with a thiol-specific derivatizing agent, ammonium 7-benzo-2-oxa-1,3-diazole-4-sulfonate (SBD), and separation of the resulting WR-1065 fluorescent derivative from endogenous thiol derivatives using HPLC.

2. Experimental

2.1. Chemicals and reagents

All chemicals were of the highest analytical grade possible. WR-1065 and its analog WR-251833, used as an internal standard, were supplied by US Bioscience, Conshohocken, PA. Both chemicals contained $<0.1\%$ chromatographically detectable impurities. Drug-free human plasma was from Biocell Laboratories, Inc., Rancho Comminguez, CA. Tri-*n*-butyl phosphine (TNBP), dimethylformamide, trichloroacetic acid, boric acid, sodium ethylenediaminetetraacetate and ammonium 7-benzo-2-oxa-1,3-diazole-4-sulfonate were obtained from Sigma Chemical Co., St. Louis, MO. All other reagents were from Fisher Scientific, Pittsburgh, PA.

2.2. Instrumentation

An automated solvent delivery system with column temperature controller (Alliance 2690], and fluorescence detector [470] (Waters Corporation, Milford, MA) was used for HPLC analyses. The column chosen for analyte separation was a Supelcosil LC-ABZ, 5 micron, 15 cm×4.6 mm ID (Supelco, Bellefonte, PA). The fluorescence excitation wavelength was 385 nm; emission was measured at 515 nm.

2.3. Sample preparation

2.3.1. Bound WR-1065 in protein pellets

Aliquots of whole blood were extracted with an equal volume of 0.5 mol/l perchloric acid/0.5 g/l EDTA, vortexed thoroughly and centrifuged at 800 *g* for 10 min to separate the protein pellets, containing bound WR-1065, from the clear supernatant, containing soluble ''free'' WR-1065. Pellets were stored at -70°C until the day of analysis. Prior to sample reduction pellets were thawed, resuspended and the pH adjusted to 7.4 with 1.0 mol/l Tris buffer, pH 10.8.

2.3.2. Total WR-1065 in plasma

Plasma samples (standards, quality controls and plasma specimens) needed no initial extraction.

2.4. Sample reduction and derivatization

Both protein-bound and total WR-1065 were analyzed using WR-1065 plasma standards and quality controls. Using a modification of the method of Garg et al. [8], sample preparation, reduction and protein precipitation procedures were carried out at 4°C, sample derivatization was performed at room temperature. Under these conditions the thiol moiety of the WR-1065 molecule remained intact. 30 μl of internal standard, 0.5 mol/l WR-251833, were added to a 300 μl aliquot of each specimen. Reduction was performed by adding 40 μl 10% TNBP in dimethylformamide to each sample, capping, vortexing and allowing to stand on ice. After 30 min samples were treated with 10% trichloroacetic acid in 1.0 m*M* EDTA, vortexed and centrifuged at 800 *g* for 10 min. A 100 μl volume of the resulting clear supernatant was added to a mixture of 20 μl of 1.55 mol/l sodium hydroxide, 250 μl of 4 m*M* EDTA in 125 m*M* borate buffer, pH 9.5 and 100 μl 7-benzo-2-oxa-1,3-diazole-4-sulfonate, 1 mg/ml, in EDTA/borate buffer and vortexed thoroughly. After addition of the

derivatizing agent samples were heated at 60°C for 60 min to facilitate complete derivatization of the thiols, dark-adapted for 10 min and chromatographed.

2.5. Chromatographic conditions

A 40 μl volume of derivatized sample was injected onto a Supelcosil LC-ABZ column which was kept at a constant temperature of 25°C. Elution of thiols was accomplished by using an aqueous isocratic mobile phase containing 10% methanol and 90% 10 m*M* ethylamine in 0.1 mol/l monochloroacetic acid, pH 2.8. Mobile phase was spurged with helium. The flow-rate was 1.0 ml/min, resulting in a column back-pressure of about 1000 p.s.i.

2.6. Data handling and calculations

For bound WR-1065 in protein pellets, the increase in ratio of peak area (WR-1065/WR-251833) for plasma standards was linear over the range of the assay (5–50 μmol/l); a calibration curve generated for each set of derivatized samples using a linear through zero (no weighting) processing method (Fig. 1(a)). For total WR-1065, where measured concentrations were higher, the calibration curve response was slightly higher than expected at the higher WR-1065 concentrations (100 μmol/l). This

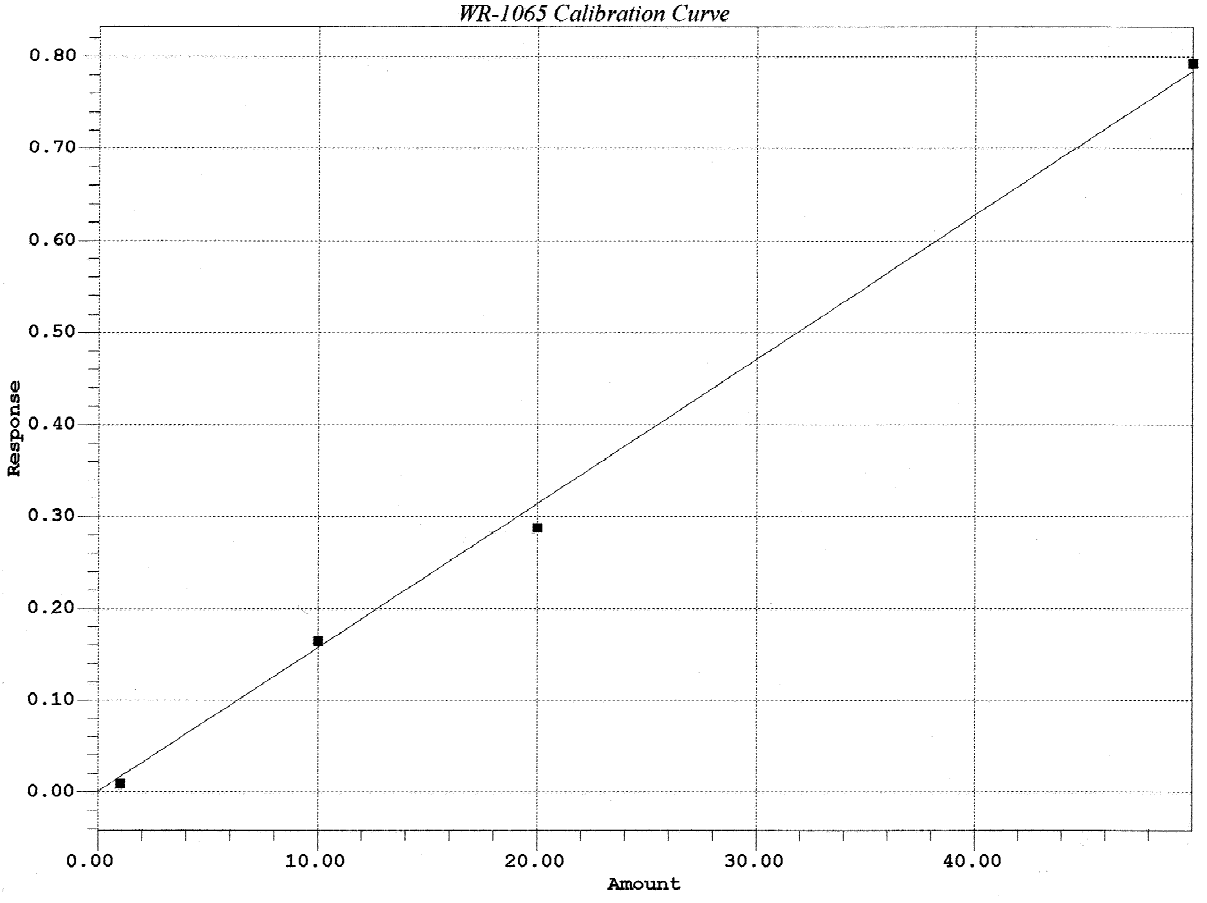

Fig. 1. (a) Calibration curve for protein-bound WR-1065 analysis: total [WR-1065] over a range of 5–50 μmol/l using a linear through zero processing method. (b) Calibration curve for total WR-1065 analysis: total [WR-1065] over a range of 5–100 μmol/l using a cubic spline processing method.

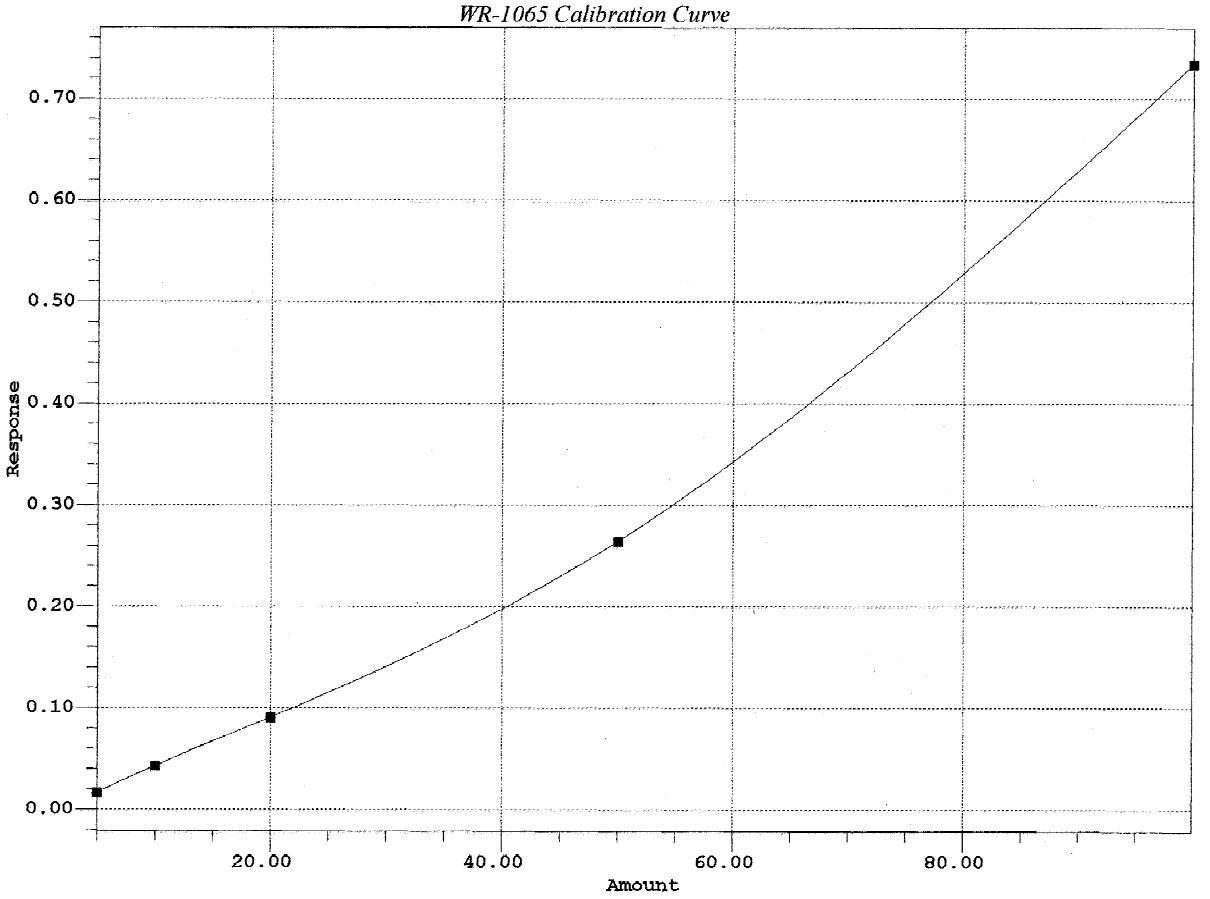

Fig. 1 (*continued*).

unexplained non-linearity [6–8] necessitated using a cubic spline (no weighting) processing method (Fig. 1(b)).

3. Results and discussion

3.1. Chromatography of total WR-1065

Separating the derivatized total WR-1065 and WR-251833 from endogenous plasma thiols presented a number of challenges. The Supelcosil LC-ABZ column demonstrated superior resolution over other LC columns (Supelcosil LC-8, LC-8DB), provided that the ionic buffer remained stable throughout the analysis. In prior published studies, phosphate buffer had been used for this type of thiol assay. Phosphate buffer was subject to bacterial contamination when kept at room temperature for extended periods of time so that the pH of the buffer was subject to change on standing. We found a monochloroacetic acid buffer to be more satisfactory. Contamination was not a problem and the pH, once carefully adjusted, remained constant throughout the analyses (often run overnight). This resulted in reproducible separation of the polar compounds of interest. Methanol (10%) was used as the organic modifier and provided better separation than acetonitrile. Bound WR-1065, as measured in a protein pellet, or total WR-1065, as measured in untreated plasma, and the internal standard, WR-251833, eluted between endogenous plasma thiols. The latter included L-cysteine, homocysteine and an unidentified thiol peak. Retention times of these polar

compounds are very sensitive to changes in mobile phase pH, so any pH adjustments should be made carefully.

3.2. Precision, accuracy and sensitivity

Between-day replications were run over a 20-day period for each method. For the protein-bound WR-1065 method quality control concentrations of 4, 40 and 60 μmol/l were spiked into plasma. Precision (SD/C.V.%) was calculated to be 1.40/41.8%, 14.4/29.2% and 10.3/16.4%, respectively; % accuracy was 85.0, 123.8 and 104.0%, respectively. Total WR-1065 measurements were stable over this time period. Tighter precision and accuracy were achieved with replication data from total WR-1065 method quality control analysis: WR-1065 concentrations of 7.0, 45.0 and 85.0 μmol/l gave between-day precision (SD/C.V.%) of 1.11/16.7%, 6.58/15.5% and 9.24/11.2%, respectively, and % accuracy of 94.7, 94.2 and 97.2%, respectively. Within-day replication is shown in Table 1. From these data we concluded that for total WR-1065 the control limits for quality control rejection should be set at ±2SDs. The theoretical limit of detection, with a signal-to-noise ratio of 10 to 1, was 44 pmoles per injected sample (1.1 μmoles/l injected sample). Chromatograms from both pre- and post-dose, after IV infusion of amifostine, 200 mg/m^2, in normal subject protein pellet samples, are shown in Fig. 2. Since amifostine does not bind to plasma proteins, no detectable amounts of the parent drug are present in protein pellet samples.

Table 1
Within-day precision (SD/C.V.%) and % accuracy for the measurement of total WR-1065

Spiked-in [WR-1065] μmol/l		Measured [WR-1065] μmol/l		
		Day 1	Day 2	Day 3
7.0	Mean:	7.77	5.27	6.95
	SD:	0.57	0.57	6.95
	C.V.%:	5.87	9.80	10.2
	% Accuracy	111.0	75.3	99.3
	n:	3	4	4
45.0	Mean:	40.5	35.2	40.9
	SD:	1.46	1.66	5.42
	C.V.%:	3.60	4.70	13.3
	% Accuracy	90.0	78.2	90.8
	n:	5	6	6
85.0	Mean:	82.5	78.7	77.3
	SD:	4.18	12.5	11.8
	C.V.%:	5.06	15.8	15.2
	% Accuracy	97.1	92.6	90.9
	n:	3	4	4

3.3. Measurement of total WR-1065 levels in the presence of amifostine

Due to the chemistry of the derivatization reaction it is necessary to determine whether there is any amifostine present in PK study plasma samples when measuring levels of total WR-1065 by this method. This is because any amifostine present in the untreated plasma samples will be hydrolyzed to WR-1065, giving an accummulative value of amifostine plus WR-1065. Since amifostine does not bind to plasma proteins [4], free levels of amifostine, as measured separately in the acid/EDTA-treated plasma supernatant, can be subtracted from the amifostine plus total WR-1065 to give the true total WR-1065 levels. Total WR-1065 quality controls containing spiked-in amifostine levels (15 and 45 μmoles/l) were run to check feasibility. The mean WR-1065 recovery was 96.9±26.5%).

4. Conclusions

We have developed a method to measure protein-bound WR-1065, using HPLC with fluorometric detection of derivatized samples. By analyzing acid-precipitated protein pellets from whole blood samples we have demonstrated that this active thiol metabolite of amifostine binds to plasma proteins in significant concentrations. These results confirmed our original radiolabelled amifostine metabolism experiments in the Balb/c mouse [5], and led to further development of a more practical method for measuring total WR-1065 in plasma. In this latter method, sample preparation is more straightforward, allowing for automated analysis with increased sample throughput. Investigations are underway to characterize the PK of WR-1065 as part of radio- and chemo-protection in clinical trials and in animal model studies.

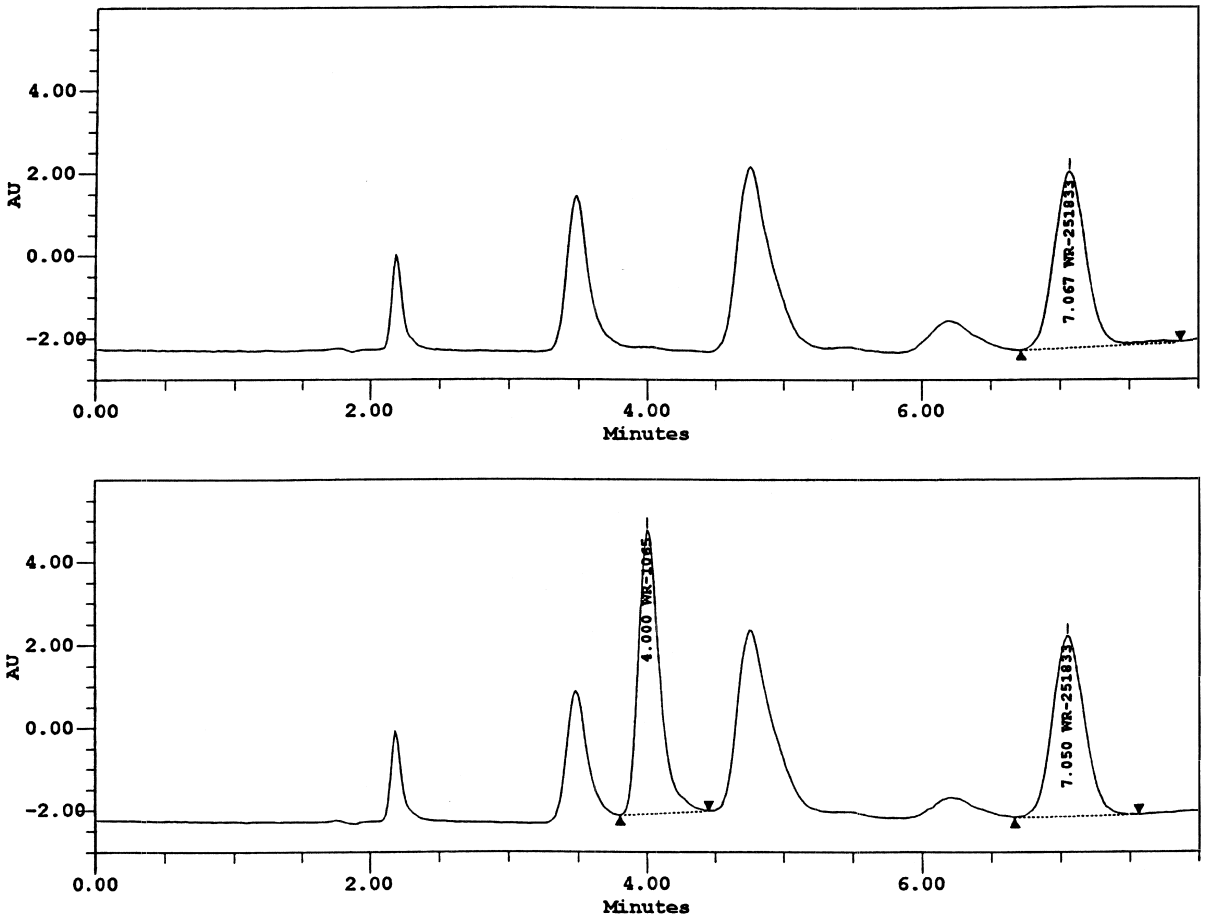

Fig. 2. Chromatogram of bound WR-1065 in normal subject protein pellet samples: (a) pre-dose ([WR-1065]=0 μmol/l) and (b) 7.5 min post-dose ([WR-1065]=103.7 μmol/l, retention time 4.0 min, internal standard, [WR-251833]=50 μmol/l, 7.0 min) after 200 mg/m^2 amifostine dose, administered as a continuous infusion over 7.5 min. Endogenous thiols L-cysteine (3.4 min), homocysteine (6.2 min) and unknown (4.7 min) do not interfere.

Acknowledgements

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