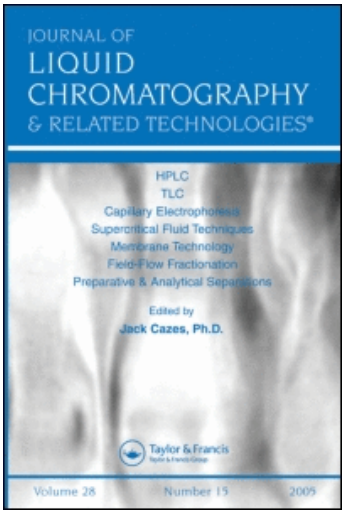

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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L. M. Shaw[a]; H. S. Bonner[a]; A. Turrisi[a]; A. L. Norfleet[a]; M. Kligerman[a]
[a] Departments of Pathology and Laboratory Medicine, Radiation Therapy and the Cancer Center of the Hospital of the University of Pennsylvania, Philadelphia, Pennsylvania

To cite this Article Shaw, L. M. , Bonner, H. S. , Turrisi, A. , Norfleet, A. L. and Kligerman, M.(1986) 'Measurement of S-2-(3-Aminopropylamino)ethanethiol (WR1065) in Blood and Tissues', Journal of Liquid Chromatography & Related Technologies, 9: 4, 845 — 859
To link to this Article: DOI: 10.1080/01483918608076673
URL: http://dx.doi.org/10.1080/01483918608076673

JOURNAL OF LIQUID CHROMATOGRAPHY, 9(4), 845-859 (1986)

MEASUREMENT OF S-2-(3-AMINOPROPYL-AMINO)ETHANETHIOL (WR1065) IN BLOOD AND TISSUE

L. M. Shaw, H. S. Bonner, A. Turrisi,
A. L. Norfleet, and M. Kligerman
Departments of Pathology and Laboratory Medicine
Radiation Therapy and the Cancer Center of the
Hospital of the University of Pennsylvania
Philadelphia, Pennsylvania 19104

ABSTRACT

A procedure for the analysis of blood and tissue specimens for WR1065, the dephosphorylated metabolite of the radioprotective drug WR2721, has been developed. The method includes the use of a perchloric acid/EDTA extraction step at 0°C followed by chromatographic analysis using a mercury/gold thin film electrochemical detection liquid chromatography system. The extraction technique was designed to assure the stability of both WR1065 and any WR2721 present in blood or tissues. Using the described chromatography conditions and an analog of WR1065, 3-(4-aminopropylamino)propanethiol (WR251833), as an internal standard the respective retention times of these two compounds are 6.2 and 8.3 minutes. Experiments showing the applicability of this method to pharmacokinetic studies of WR2721 and WR1065 and to investigation of the kinetics of WR2721 hydrolysis in biological fluids such as stomach juice are described.

INTRODUCTION

WR2721 is an experimental drug that provides significant radioprotection to many normal tissues but provides little or no protec-

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tion to many experimental tumors (1,2). Several reports have shown that WR2721 provides significant protection against the toxic effects of the chemotherapeutic drugs, cisplatin and cyclosphosphamide (3,4). A third pharmacologic action of WR2721 is its hypocalcemic effect (5). The single dose phase I trials of WR2721 as a radioprotector and chemoprotector have been completed (6,7,8) and the drug has been shown to be effective in reducing the serum calcium concentration in a patient with hypercalcemia secondary to parathyroid cancer (9).

WR1065, the dephosphorylated free sulfhydryl metabolite of WR2721, is generally regarded as the active form of the parent drug and/or the precursor of active form(s) of the drug (i.e. the symmetrical disulfide of WR1065 or mixed disulfides of the latter and endogenous sulfhydryl compounds such as cysteine, glutathione and proteins). Thus in order to establish the optimal dosage and time sequence of the protector and treatment in patients it is essential to be able to measure both WR2721 and WR1065 in blood and tissues. Although methods for measuring WR2721 and WR1065 have recently been described (10,11,12, 13) there are no existing methods for satisfactorily measuring both WR2721 and WR1065 in blood and tissues. Efforts to achieve this goal have been hampered by the fact that each compound has stability problems that are different. Thus, the challenge has been to devise a blood and tissue preparation method that assures stability for both WR2721 and WR1065 and which is compatible with the subsequent chromatography methods of analysis. Here we report a sample preparation method that is simple and which assures the stability of WR2721 and WR1065 in biological samples.

The prepared specimens are analyzed directly with an HPLC method using a mercury/gold amalgam electrochemical detector at the selective potential of + 0.15 volts.

MATERIALS AND METHODS

Apparatus

A Bioanalytical Systems LC-304 liquid chromotagraph including a dual piston pump operated at 3,500 psi and a mercury/gold electrochemical detector was used as previously described (11). Column temperature was maintained at 25°C with a temperature jacket. All teflon tubing was replaced with stainless steel to exclude oxygen. The column used for these studies was the BAS Biophase ODS 5u (4.6 x 250 mm). The mobile phase was continuously purged with nitrogen to remove dissolved oxygen.

Chemicals

S-2-(3-aminopropylamino)ethanethiol (WR1065), S-2-(3-aminopropylamino)ethylphosphorothioate (WR2721), and 3-(4-aminopropylamino) propanethiol (WR251833) were supplied to us by Dr. Lawrence Fleckenstein of the United States Army Medical Research and Development Command at Walter Reed Army Institute of Research. Acetonitrile and methanol were obtained from Fischer Scientific (King of Prussia, PA) and sodium octyl sulfate was from Eastman Kodak Co. (Rochester, NY). All other reagents used were of the highest analytical grade available.

Sample Preparation and Chromatography

Unless otherwise noted specimens were processed at 0°C by adding an aliquot of a solution of 1 mol/L perchloric acid in 2.7 mmol/L disodium EDTA to an aliquot of the sample to be analyzed in the ratio 2 to 5, respectively. Samples that contained protein were centrifuged in the cold (4°C) for 15 min in order to prepare the supernatant fraction for injection into the chromatograph. Twenty microliter aliquots of the protein-free specimens were injected onto a Biophase 5 u octadecylsilane column (250 x 4.6 mm) that was maintained at 25°C with a constant temperature jacket. Elution of WR1065 at 6.2 min and the internal standard WR251833 at 8.3 min was achieved isocratically using a 40% (v/v) methanol/water mobile phase containing 0.1 mol/L monochloroacetic acid and 1.0 mmol/L sodium octylsulfate, pH 3.0, at a flow rate of 1.3 mL/min.

RESULTS AND DISCUSSION

Detection, Linearity and Sensitivity

A linear sweep voltammogram of a solution of WR1065 is displayed in Figure 1. The voltammogram shows that the sulfhydryl is oxidized at the surface of the mercury/gold amalgam electrode at a low potential. This is consistent with the observed electrochemical properties of other sulfhydryl compounds such as glutathione and cysteine (14) and shows that the mercury/gold electrode set at a potential of + 0.15 V with respect to a Ag/AgCl reference electrode is a suitable detector for HPLC analysis of WR1065. A typical chromatogram showing detector response versus elution time for WR1065

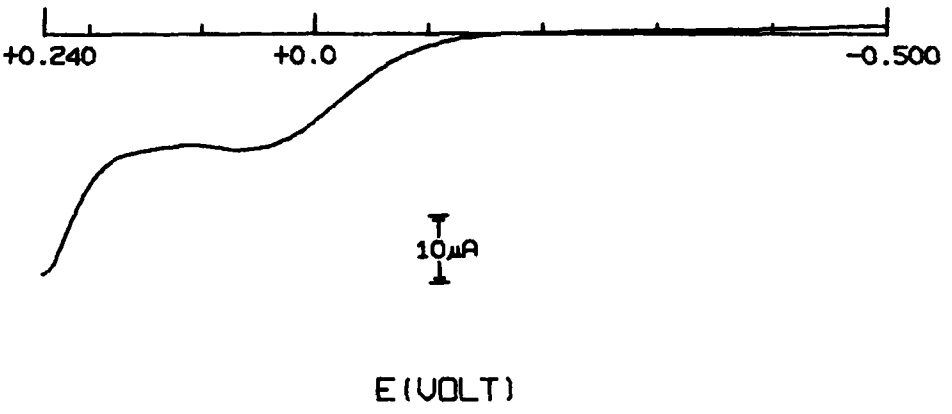

FIGURE 1. A linear sweep voltmmogram of 4 mmol/L WR1065 in an aqueous solution of 0.1 mol/L monochloroacetic acid and 1.5 mmol/L sodium octylsulfate, pH 3, using a mercury/gold electrode.

and WR251833 is shown in Figure 2. The ratio of peak heights of varying concentrations of WR1065 to that of the internal standard WR251833, at a concentration of 100 umol/L in an aqueous solution containing 10 mmol/L Tris, pH 7.4, and 1 g/L sodium EDTA, was determined. The increase in the ratio of WR1065 peak heights to that of the internal standard was linear over the WR1065 concentration range of 2.5 to 250 umol/L (R^2 = 0.998). The limit of sensitivity was determined: 2 pmol per injected sample (100 nmoL/L of sample).

Sample Preparation

The use of the perchloric acid/EDTA solution at 0°C for sample preparation is critical to the analysis of WR1065. It has been shown for sulfhydryl compounds such as glutathione that at neutral pH autoxidation occurs in deproteinized samples (15). Further if

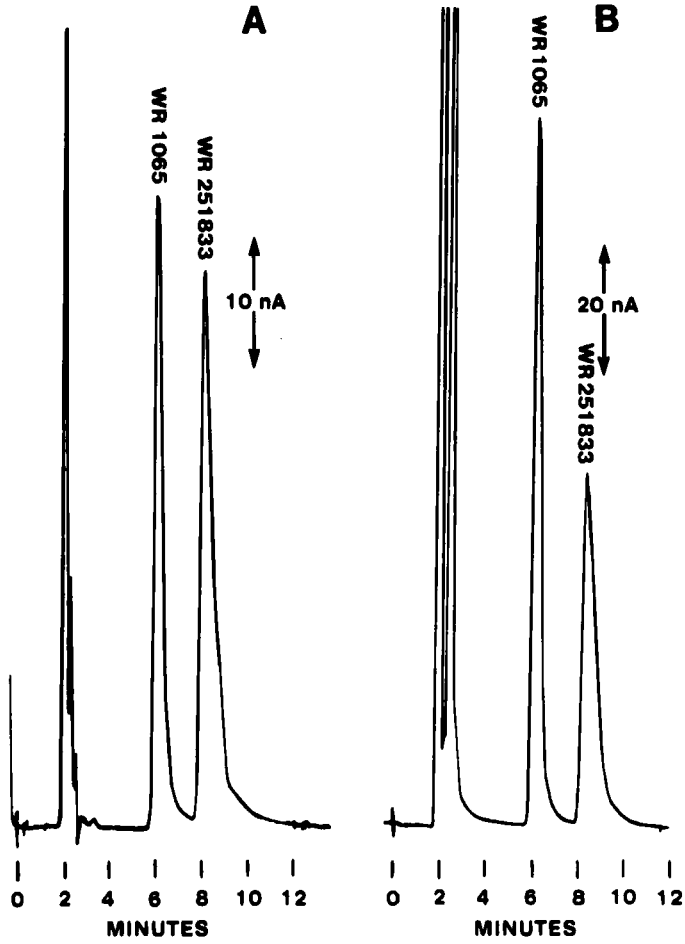

FIGURE 2. A. Chromatogram of a mixture of a solution of 100 umol/L WR1065 and 100 umol/L WR251833 and perchloric acid/EDTA solution in the proportions described in Materials and Methods. Sensitivity is 100 nA full scale. B. Chromatogram of perchloric acid/EDTA extract of normal human blood to which was added 200 umol/L WR1065 and 100 umol/L WR251833. Sensitivity is 200 nA full scale.

TABLE 1

Decrease of WR1065 Concentration in Whole Blood at 0°C

Incubation time (min.)	WR1065 Concentration (umol/L)	Percent Original Concentration, %
0	205 (3.06)[a]	100
30	125 (0.5)	61
60	106 (1.0)	52
120	101 (2.8)	49
180	85 (0.7)	41

Human blood from a healthy subject was collected in EDTA vacutainer tubes. 3.0 mL of a freshly prepared ice-cold solution of 1.0 g/L disodium EDTA, 10 mmol/L Tris, pH 7.4, containing 1.33 mmol/L WR1065 and 0.67 mmol/L WR251833 was added to 17 mL of the ice cold blood sample.

At the indicated times a 1 mL aliquot of the blood sample was taken and immediately added to 0.4 mL of ice-cold 1 mol/L perchloric acid, 2.7 mmol/L disodium EDTA solution. Further sample processing and chromatographic analysis were performed as described in Materials and Methods.

[a] Each result is the average of triplicate determinations. Values in parentheses are standard deviations.

glutathione is added to human plasma and incubated at 37°C, it rapidly disappears to produce glutathione disulfide (16). We have found that WR1065 added to normal human blood and incubated at 0°C rapidly disappears, presumably, to form disulfide and mixed disulfide products (Table 1). This process is prevented by the immediate treatment of blood with the perchloric acid/EDTA solution as described above. It has been shown that autoxidation of sulfhydryl compounds is minimal at low pH and that precipitation of blood proteins removes any possible enzymes that could catalyze sulfhydryl oxida-

TABLE 2

Determination of the Accuracy of the HPLC Method

	Whole Blood		Liver	
Spiked-in WR1065 Conc. (umol/L)	Mean Measured Concentration	Percent Deviation (D)	Mean Measured Concentration	Percent Deviation (D)
5	5.01(0.084)	0.2	4.87(0.17)	-2.6
50	53.6(1.45)	7.2	57.9(1.56)	15.8
100	107(1.92)	7.0	102(0.44)	2.0
200	201(3.91)	0.5	187(3.03)	-6.5
	Average % D = 3.7		Average % D = 6.7	

To ice-cold aliquots of a pool of blood collected from a healthy volunteer were added the indicated WR1065 concentrations. Using the procedure described in Table 1 the blood specimens were processed immediately after addition of the ice-cold WR1065 solution. The same spiking-in procedure was used to determine the accuracy of the WR1065 HPLC analysis method for mouse liver homogenate. The latter was prepared from normal mice by homogenizing a mixture of 1 g of liver in a total volume of 5 mL of 1 g/L disodium EDTA, 10 mmol/L tris, pH 7.4. The concentration of WR251833, the internal standard, was 100 umol/L in all specimens. Each measured concentration is the mean of 4 determinations and the numbers in parentheses are standard deviations.

tion (15). Since the disappearance of WR1065 in blood at 0°C is so rapid it is essential to immediately treat blood samples containing WR1065 with ice-cold perchloric acid/EDTA solution. When processed in this way we obtained very good recovery of WR1065 that had been added to human blood or mouse liver homogenate (Table 2).

Thus it is essential that these low pH conditions be used in order to obtain accurate WR1065 values. Since measurements of WR1065 are often made in the presence of WR2721 it is important to

establish whether or not the perchloric acid/EDTA solution produces significant hydrolysis of the latter at 0°C. The rate of non-enzymatic hydrolysis of WR2721 is strongly dependent on pH (10,17,18) and temperature (18). The rate increases with decreasing pH and decreases with decreasing temperature. We determined the pseudo-first-order rate constant for hydrolysis of WR2721 in the perchloric acid/EDTA solution at 0°C and obtained a value of 0.108 x 10^{-3} min^{-1} (Table 3). Thus the rate of hydrolysis of WR2721 under these conditions would be 0.011% per minute or about 0.6% per hour. This very slight rate of hydrolysis will not produce significant false increases in WR1065 concentration for the duration of a workday (~4.2% over 7 hours). As shown in Table 3 the WR2721 pseudo-first-order hydrolysis rate constant in the perchloric acid/EDTA solution at 25°C, 8.0 x 10^{-3} min^{-1}, is 74 times that at 0°C. At 25°C WR2721 hydrolysis would proceed at a rate of 0.8% per minute. Thus, in order to preclude production of significant quantities of WR1065 from WR2721 using the described sample preparation procedure it is essential to maintain samples at 0°C at all times prior to injection onto the HPLC column.

WR1065 blood concentration in a patient given multiple WR2721 doses

The applicability of the described techniques to pharmacokinetic studies is illustrated in Figure 3. WR2721 and WR1065 blood concentrations were plotted versus time in a patient who was given 5 multiple intravenous injections of 150 mg/M^2 WR2721. The first 4 were given every 4 minutes and the fifth and last was administered 3

TABLE 3

Pseudo-First-Order Rate Constants for Hydrolysis of WR2721

	Temp.	pH	First-order rate const. x 10^3 (min^{-1})	T1/2
0.4 mol/L $HClO_4$, 1 mmol/L sodium EDTA	0°C	0.80	0.108	107 hr
0.4 mol/L $HClO_4$, 1 mmol/L sodium EDTA	25°C	0.80	8.0	87 min
Normal human stomach juice	37°C	1.74	22.74	30.5 min

The pseudo-first-order rate constant for hydrolysis of WR2721 was obtained from WR1065 concentrations measured by the described electrochemical liquid chromatography method. For each rate constant determination WR1065 concentrations were measured at 9 consecutive 10 minute intervals for the 25°C study in perchloric acid/EDTA, 4 consecutive 1 hour intervals and at 24 hours for the 0°C study in perchloric acid/EDTA and 5 consecutive 20 minute intervals for the 37°C study in stomach juice. In each study the original concentration of WR2721 was 200 umol/L and one aliquot of the reaction mixture was hydrolyzed completely to WR1065 by incubation of 45°C for 2 hours. The pseudo-first-order rate constant for the hydrolysis reaction was obtained from the slope of the plot of the natural logarithm of the difference between WR1065 produced at each time point and that produced by complete hydrolysis as a function of time.

minutes after the fourth dose. The data shows that WR1065 concentration in blood increased steadily during the time interval of the first four doses, reached a plateau concentration of about 100 umol/L then decreased to a concentration of 35 umol/L 60 minutes after the first dose was given. WR2721 concentration declined rapidly after reaching a concentration of 1200 umol/L 30 seconds after administration of

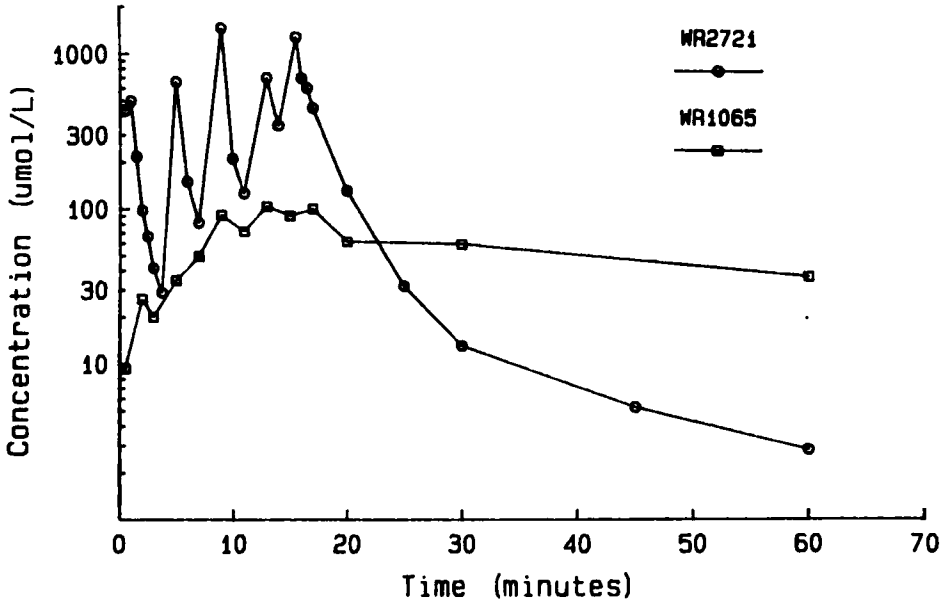

FIGURE 3. A plot of the log WR2721 and WR1065 blood concentrations versus time in a patient who was given four bolus injections of 150 mg/M^2 WR2721 at 4 minute intervals and a fifth bolus injection 3 minutes after the fourth dose. Each point is the average of duplicate determinations.

the fifth bolus dose. 10 minutes after the last dose the WR2721 concentration became lower than that of WR1065. Thus WR1065, the free sulfhydryl metabolite of WR2721, appeared in the bloodstream of the patient shortly after administration of WR2721 and remained in blood at higher concentrations than that of WR2721 for a longer period of time. It has previously been shown that WR1065 appears in various tissues shortly after intravenous administration of WR2721 to mice (12). Furthermore tissue concentrations of WR2721 have been shown by us to be, in general much lower than those of WR1065 in mice

shortly after administration of WR2721 (19). Thus the rapid decrease in WR2721 concentration and rapid appearance of WR1065 in the patient's bloodstream is consistent with the observations of the fast rate of appearance of WR1065 in mouse tissues after WR2721 administration.

It is very likely that WR1065 reacts, in vivo, with other endogenous compounds such as cysteine, glutathione and certain proteins to form mixed disulfides. Future studies will be required to identify and quantitate these.

Production of WR1065 from WR2721 in human stomach juice

It has previously been noted that after oral administration of WR2721 there is a rapid and significant loss of the radioprotective activity of the compound (10). This has been presumed to result from acid-catalyzed hydrolysis of WR2721 to produce WR1065. The latter then presumably was further metabolized to inactive compounds resulting in a loss of radioprotective activity. Our data in Table 3 show that the rate constant for WR2721 hydrolysis in a specimen of normal human stomach juice (pH 1.74) is 22.74×10^{-3} min^{-1}. The half-life for hydrolysis of WR2721 in the stomach juice sample is, therefore, 30.5 minutes. Thus there is a fairly rapid rate of hydrolysis of WR2721 in stomach juice. Although some intact WR2721 might reach the small intestine in subjects given the drug orally it is very likely that the remaining drug would be rapidly dephosphorylated at the surface of intestinal microvilli by alkaline phosphatase. The latter plasma membrane enzyme is present in high concentrations

in small intestinal microvilli (20) and it has been shown to readily catalyze the dephosphorylation of WR2721 (11).

ACKNOWLEDGMENTS

This work was supported by the National Cancer Institute Grant CA-30100 from the Department of Health and Human Services and by an institutional grant from the American Cancer Society. We thank Drs. John Glick, Ronald Shoup and Robert Van Etten for helpul discussions of this work and Drs. Daryll Brown and Ann Ouyang for the mouse liver and normal human stomach juice specimens respectively.

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