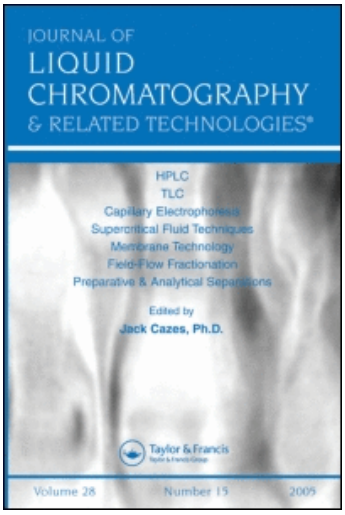

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Detection and Measurement of the Disulfide WR33278 $[NH_2(CH_2)_3NHCH_2CH_2S-]_2$ in Blood and Tissues

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DETECTION AND MEASUREMENT OF THE DISULFIDE WR33278 $[NH_2(CH_2)_3NHCH_2CH_2S-]_2$ IN BLOOD AND TISSUES

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ABSTRACT

A liquid chromatographic method employing an in-series dual mercury/gold amalgam electrochemical detector has been developed for measurement of the symmetrical disulfide metabolite WR33278 $[NH_2(CH_2)_3NHCH_2CH_2S-]_2$ of the radioprotector WR2721 in blood, tissues and biological fluids such as urine. The problem of adsorption of this disulfide to the octylsilane reverse phase column was overcome by including 10.7 mmol/L ethylamine in the mobile phase. The retention times for WR33278 and WR183159 $[CH_3NH(CH_2)_3NHCH_2CH_2S-]_2$, the internal standard, are approximately 3.9 and 11 minutes, respectively. Linearity of the method was to 1000 umol/L. This method has good precision and accuracy and has a sensitivity limit of 0.2 umol/L. Investigation of the metabolism and pharmacokinetics of WR33278 in patients and experimental animal models, after administration of WR2721, using the described chromatographic conditions is currently underway.

INTRODUCTION

WR2721, an experimental radio-and chemoprotective drug (1-7) and hypocalcemic agent (8,9) is rapidly cleared from the

bloodstream (3,10) by a variety of normal tissues. Uptake is presumed to be mediated by plasma membrane alkaline phosphatase (11-13). According to this postulated pathway WR2721 reaches alkaline phosphatase-rich plasma membranes via circulating blood plasma. WR2721 is then hydrolyzed at the surface of such plasma membrane sites by alkaline phosphatase to produce the free sulfhydryl metabolite WR1065. The latter then penetrates cell membranes to reach critical intracellular sites, providing, by an unknown mechanism(s), its protective or other pharmacological actions. The degree of radioprotection provided by WR2721 varies from tissue to tissue which could result, at least partially, from the observed differences in the rate of intracellular accumulation and/or further metabolism of WR1065 (14). Pursuit of the investigation of the metabolism of WR1065 requires development of a specific and sensitive method for measurement of its symmetrical disulfide, WR33278. Here we report the development of a reverse phase liquid chromatography method using an in-series dual mercury/gold amalgam electrochemical detector and a perchloric acid/EDTA sample preparation step that affords reliable measurement of WR33278 in biological fluids and tissue.

MATERIALS AND METHODS

Apparatus

A Bioanalytical Systems LC-304 liquid chromatograph with tandem LC-4B amperometric controllers was modified to exclude oxygen from the system. All teflon tubing was replaced with stainless steel.

A dual piston pump operated at 3,300 psi and a dual, in series, mercury/gold amalgam electrochemical detector was used as previously described (15). W_1, the upstream electrode was held at a constant potential of -1.000 V while W_2, the downstream electrode, was held at a potential of + 0.15 V. Temperature of the column was maintained at 25°C with a temperature jacket. The column used for these studies was the BAS Biophase Octyl 5u (4.6 x 250 mm). The mobile phase was continuously purged with nitrogen to remove dissolved oxygen.

Chemicals

WR33278 $[NH_2(CH_2)_3NHCH_2CH_2S-]_2$ and WR183159 $[CH_3NH(CH_2)_3NHCH_2CH_2S-]_2$ 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 Fisher Scientific (King of Prussia, PA), and ethylamine was from Sigma Chemical Co. (St Louis, MO). All other reagents used were of the highest analytical grade available.

Sample Preparation and Chromotrography

All specimens were processed at 0°C by adding an aliquot of a solution of 0.56 mol/L perchloric acid in 1.54 mmol/L disodium EDTA to an equal volume aliquot of the sample to be analyzed. These mixtures were then centrifuged in the cold (4°C) for 15 min in order to prepare the supernatant fraction for injection into the chromotograph. Twenty microliter aliquots of the protein-free

specimens were injected onto a Biophase octyl 5u column (250 x 4.6 mm) that was maintained at a constant temperature of 25°C with a temperature jacket. Elution of WR33278 at 3.9 min and the internal standard WR183159 at 11.0 min was achieved isocratically using an aqueous mobile phase that contained 10.7 mmol/L ethylamine, 1% methanol and 0.1 mol/L monochloroacetic acid, pH 3.0, at a flow rate of 2 mL/min.

RESULTS AND DISCUSSION

Detection, Linearity and Sensitivity

A cyclic voltammogram of a solution of WR33278 is shown in Figure 1. The voltammogram shows that WR33278 is reduced at the surface of the mercury/gold amalgam electrode at a potential of -810 mV which is similar to that observed for other disulfides such as glutathione disulfide (15). The free sulfhydryl compound produced by the reductive step would be WR1065 which is then oxidized at the electrode surface at a potential of + 0.15 V. This is consistent with the reported oxidation of WR1065 at the surface of a mercury/gold amalgam electrode (16). These data show that the in-series dual mercury/gold electrode with W_1, the upstream electrode, set at -1.000 V and W_2, the downstream electrode set at + 0.15 V with respect to a Ag/AgCl reference electrode is an appropriate detector for HPLC analysis of WR33278.

Initial attempts at chromatography using the mobile phase previously recommended for glutathione disulfide analysis (15) were unsuccessful due to significant adsorption of both WR33278 and the internal standard to the column. The problem of adsorption of

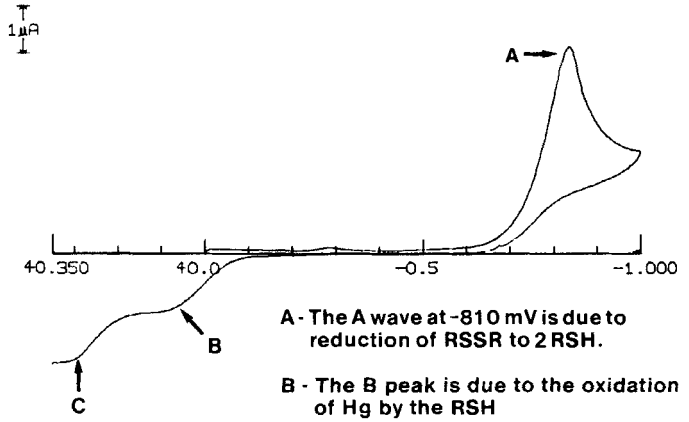

FIGURE 1

disulfide compounds to reverse phase HPLC columns was recently described by Perrett and Rudge (17). We found that the adsorption problem was overcome by replacing sodium octylsulfate with ethylamine in the mobile phase. The amino group of ethylamine is presumed to complex with free hydroxyl residues in the solid phase thus eliminating any interactions of the terminal amino groups of WR33278 with these moieties, thereby preventing adsorption to the octylsilane column. A typical chromatogram showing detector response versus elution time for WR33278 and the internal standard WR183159 is displayed in Figure 2. The ratio of peak heights of varying concentrations of WR33278 to that of the internal standard WR183159, at a concentration of 100 umol/L in drug free human

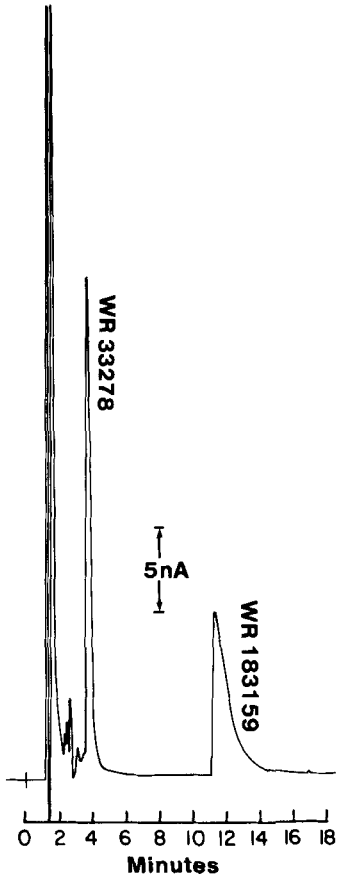

FIGURE 2

plasma was determined. The increase in the ratio of WR33278 peak heights to that of the internal standard was linear over the WR33278 concentration range of 1.0 to 1000 umol/L (R^2 = 0.999). The limit of sensitivity, signal to noise ratio of 3 to 1, was 4 pmol per injected sample (200 nmol/L of sample).

Sample Preparation

It was shown previously (16) that the immediate treatment of blood samples with the perchloric acid/EDTA solution at 0°C for sample preparation is critical for prevention of oxidation of WR1065 to disulfides including its symmetrical disulfide WR33278. WR2721 hydrolysis under these acidic conditions is prevented by maintenance of the temperature at 0°C (16,18). This step is also critical in the measurement of WR33278, as well, since the latter is determined in the presence of considerable quantities of WR1065 in blood and tissues of patients and animals after administration of the parent drug WR2721. Furthermore the use of the perchloric acid/EDTA step prevents any possible disulfide interchange reactions that could occur at physiologic pH which would be another potential artefact in the measurement of WR33278 in biological samples. It has been noted that certain acids such as perchloric, trichloroacetic and metaphosphoric alter the reduced glutathione: oxidized glutathione ratio in the sample preparation step for certain tissues (19) presumably due to conversion of the reduced to the oxidized sulfhydryl compound. We therefore tested for the possible conversion of WR1065 to WR33278 by incubating 1 mmol/L WR1065 in the perchloric acid/EDTA solution described above at 0°C. Using the described WR33278 HPLC method we detected no WR33278 formation in samples taken at incubation times of 1, 2, 3.5, 5.5 and 24 hours. Thus within the sensitivity limit of the described method (< 0.2 umol/L) perchloric acid did not catalyze the production of WR33278 from WR1065. When the described sample

TABLE 1

The Precision and Accuracy of the HPLC Method

Spiked-in WR33278 Conc. umol/L	Mean Measured Conc.[a]	Precision (SD)CV[b]	Percent Deviation (D)[c]
5	3.98	(0.07) 1.8%	-20.0
10	9.7	(0.14) 1.5%	- 3.0
50	56.2	(0.66) 1.2%	12.4
100	104	(1.44) 1.4%	4.0
500	473	(8.33) 1.8%	- 5.4

Average % deviation = 8.96 (The average of the sum of the absolute values of D).

[a] The mean measured concentrations obtained with the HPLC method for each of four blood samples at each WR33278 concentration using individually spiked specimens. The internal standard was 100 umol/L WR183159.

[b] (SD)CV, the standard deviation and coefficient of variation of the quadruplicate determinations.

[c] (D) is the percent deviation of the mean measured concentration from the spiked in concentration.

preparation method was used we obtained reproducibly very good recovery of WR33278 that had been added to human blood (Table 1). We further tested the accuracy of the method by determining recovery of WR33278 added to a mouse liver homogenate. For added-in concentrations of 10, 50, 100 and 500 umol/L WR33278 the percent recovery values were 86.7, 94.4, 108 and 89.8%, respectively. The mean percent deviation calculated from those data is 9.3%, which is comparable to the value of 8.9% obtained for spiked blood samples (Table 1).

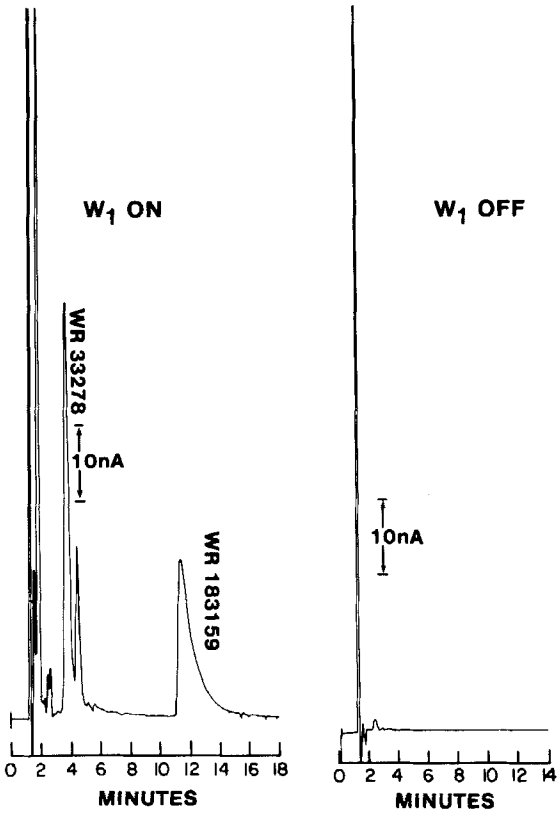

FIGURE 3

The applicability of the described method for WR33278 measurement is illustrated in Figure 3 which shows a chromotograph of a urine specimen collected from a patient one hour after administration of a single dose of WR2721 (5.02 mmoles). 243.2 umol/L of WR33278 was detected in this specimen which, after correction for the total volume of the collected specimen of 325 mL represents 1.6% of the WR2721 dose. When W_1 the upstream electrode

is turned off the WR33278 and WR183159 peaks disappear as expected for disulfides. Also an unidentified peak that eluted later than WR33278 at 4.3 min was found. Since this peak also disappeared when W_1 was off it must be an endogenous disulfide since the latter class of compound, but not reduced sulfhydryl compounds, require reduction at the W_1 electrode surface first in order for detection at the oxidizing W_2 downstream electrode to be possible. We have not observed any endogenous interfering substances that co-elute with either WR33278 or WR183159 in control blood and urine specimens obtained from patients prior to administration of WR2721. Investigation of the metabolism and pharmacokinetics of WR33278 in patients and mice after administration of WR2721 is underway in our laboratory utilizing the described electrochemical HPLC method.

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