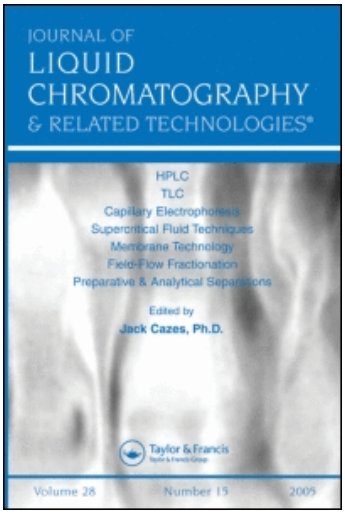

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HPLC Assay for S-2-(3-Aminopropylamino)ethyl Phosphorothioate (WR 2721) in Plasma

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HPLC ASSAY FOR S-2-(3-AMINOPROPYLAMINO)ETHYL PHOSPHOROTHIOATE (WR 2721) IN PLASMA

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ABSTRACT

A specific HPLC assay has been developed for determination of the radioprotective drug WR 2721. The method is based on precolumn derivatization of plasma with fluorescamine, separation with a C-18 cartridge and detection by fluorescence. An external standard was used for calibration, and values were adjusted based upon recovery of added ^{14}C-labeled WR 2721. WR 2721 had a retention time of about 13 minutes using a mobile phase of acetonitrile/water (22:78), 0.01 M in dibutylammonium phosphate, at a flow rate of 2 mL/min. Sensitivity of the assay was characterized to 2 μg/mL, and detector response was linear over the range of 2 to 1100 μg/mL. The assay requires 90 μL of plasma and has a total chromatography time of about 45 minutes. 2-(3-Aminopropylamino)ethanethiol (WR 1065) and bis-[2-(3-aminopropylamino)ethyl]disulfide (WR 33278), metabolites of the drug, and a variety of primary amines were shown not to interfere with the assay. Suitability of this assay for pharmacokinetic studies was demonstrated in preliminary experiments with a beagle dog.

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INTRODUCTION

The chemical radioprotector S-2-(3-aminopropylamino)ethyl phosphorothioate (I) has been the object of intense study by research groups of the U.S. Army and the National Cancer Institute. An Army program established in 1959 found that the drug [labeled Walter Reed (WR)-2721] protects various tissues from radiation (1). It was also demonstrated that certain solid animal tumors are not protected, which suggested its use in radiotherapy (2).

$$H_2N—CH_2CH_2CH_2—NH—CH_2CH_2—S—R$$

I, WR 2721, R = $PO(OH)_2$
II, WR 1065, R = H
III, WR 33278, R = $SCH_2CH_2NHCH_2CH_2CH_2NH_2$

It is believed that in the body the drug is transported intact into the tissue where it is enzymatically cleaved to yield the thiol II (WR 1065) (3). Free radicals produced by radiation are then scavenged by interaction with the sulfhydryl (SH) group of II (4).

A major shortcoming of WR 2721 is that its activity after oral dosing is severely limited--probably due to premature cleavage of the phosphate group under the acidic conditions encountered immediately after oral dosing (5).

Attempted bioassay of WR 2721 has been hampered by several serious obstacles. The compound is acid-labile, has no convenient chromophore, has essentially no solubility in organic solvents and is extremely polar, capable of existing as a dipolar ion or a bis-dipolar ion. Lack of solubility precludes its extraction from biological fluids and its polarity places limits on the types of chromatographic systems which might be used to separate it from endogenous materials. To facilitate

pharmacokinetic studies of the drug, an analytical procedure capable of efficiently processing large numbers of samples is also an important consideration. An HPLC method which overcomes the stated problems and is capable of handling multiple samples is presented in this paper.

MATERIALS AND METHODS

Instrumentation

A Waters Associates Model 244 Liquid Chromatograph equipped with a Model 420 AC Fluorescence Detector and Data Module was employed. Excitation wavelength was 395 nm and emission wavelength was >460 nm. Samples were injected using a Rheodyne Model 7125 Injector fitted with a 20- or 50-μL loop. Separations were carried out on a Waters Associates RCM-100 Radial Compression Module fitted with a 100-mm x 8-mm cartridge filled with 5-μm spherical C-18 packing. The analytical column was protected with a Whatman, Inc. guard column filled with Waters Associates CoPell C-18 packing. The mobile phase was acetonitrile/water (22:78), 0.01 M in dibutylammonium phosphate (pH ~3) at a flow of 2.0 mL/min.

Reagents

Acetonitrile was purchased from Burdick and Jackson Laboratories, Inc. Dibutylamine and fluorescamine were obtained from Aldrich Chemical Company. Concentrated (1 M) solutions of dibutylammonium phosphate were prepared by titrating 12.9 g of dibutylamine to pH 2.5 with phosphoric acid and diluting to volume. Fresh solutions containing fluorescamine (5 mg/mL) were prepared weekly using reagent grade acetone which had been stored over 4A molecular sieves. WR 2721 trihydrate, Lot AU-BJ 09506AJ-68-2, was furnished in >99.0% purity by the Walter Reed Army

Institute of Research. Radiolabeled WR 2721, S-[2-(3-aminopropylamino)ethyl-1,2-^{14}C]phosphorothioate was obtained from Research Triangle Institute, Lot 3874-52; its specific activity was 86.0 μCi/mg and reported purity was >97.0%.

Standards containing WR 2721 were prepared by dissolution in 0.05 M sodium borate-potassium chloride pH 10 buffer.

Sample Preparation

Plasma (90 μL), 50 μL of a solution of the radiolabeled internal standard (1.40 μg/mL) and 160 μL of 0.05 M sodium borate-potassium chloride buffer were placed in a polyethylene vial and, while the mixture was being agitated using a vortex mixer (American Scientific Products), 200 μL of the fluorescamine reagent was added. After mixing for 60 s, the mixture was treated with an additional 200 μL of fluorescamine reagent and agitation was continued for 20-30 s. The resulting mixture was centrifuged at 1500 rpm for three minutes and an aliquot of the supernatant was injected onto the HPLC column. Each frozen sample was individually thawed, derivatized and immediately injected because both WR 2721 and its derivative decompose in plasma at room temperature.

Internal Standard

Plasma samples were spiked with increasing amounts of WR 2721, mixed with internal standard, derivatized and injected as described above. The following concentrations were used to construct a standard curve: 0, 1.89, 6.33, 11.9, 56.3, 112, 279, 556, 834, and 1110 μg/mL. The curve was constructed by fitting a regression line to the peak area versus concentration data after correction for recovery. Recovery was determined by adding 50 μL of a solution containing ^{14}C-labeled WR 2721 (activity 334 dpm/μL) to each sample vial prior to derivatization. After

derivatization, a 20- or 50-μL sample was removed from the reaction vial, mixed with scintillation fluid and counted using a Packard Tri-Carb Model 4530 scintillation counter. Similarly, either a 20- or 50-μL sample was analyzed using HPLC. Twelve 1-mL fractions of column effluent were collected starting three minutes prior to the elution time of WR 2721 and continuing for six minutes. Each vial, after addition of scintillation fluid, was counted for 10 minutes, and the recovered radioactivity was determined by summing the disintegrations observed in those fractions with activity greater than the background level. Recovery of WR 2721 was then determined for each assay using the expression: % recovery = (dpm recovered x 100)/dpm added. All calculations were corrected for contributions due to background. Counting efficiencies were determined via automatic external standardization. Recoveries obtained were between 35% and 55% (mean = 48%, n = 58).

Animal Dosing Experiments

A healthy, one-year old, AKC-registerable, male beagle dog weighing 12.7 kg was used in pilot dosing experiments to test the analytical method. The animal was dosed intravenously in the cephalic vein with a 0.9% saline solution containing 1.9 g (150 mg/kg body weight) of WR 2721. The infusion required two minutes. Blood samples (3 mL) were withdrawn into an EDTA Vacutainer® from a cannula placed in the jugular vein. Each sample was immediately chilled in an ice/water bath and then centrifuged. A 90-μL aliquot of the separated plasma was added to 50 μL of the internal standard, the mixture was agitated using a vortex mixer and was then quick-frozen in a dry ice/alcohol bath. Samples were stored at -20°C until time of analysis when they were thawed at room temperature and immediately treated as described in the Sample Preparation section. The drug was shown

to be stable in plasma up to about 30 days when stored at -20°C or lower, but was unstable at room temperature.

RESULTS AND DISCUSSION

Derivatization

Major obstacles in the development of the assay were the instability of WR 2721 in acidic media and its lack of a suitable chromophore. A pH-hydrolysis rate profile (Figure 1) demonstrates the lack of stability under acidic conditions. The data were generated by measuring the rate of appearance of WR 1065 (6) and calculating the rate of disappearance of WR 2721.

In simple aqueous systems underivatized WR 2721 has been analyzed by HPLC using separation on a Whatman PAC column (7) and UV detection at 204 nm, but this procedure was unsuitable for plasma analysis. Derivatization of the drug with fluorescamine allowed detection in the picomole range and at the same time modified its chromatographic behavior such that it was retained on reverse-phase columns.

It was found that precision was remarkably improved by a two-stage derivatization procedure (see Methods section). This may be attributed to the effect of the acetone solvent which, by protein denaturization, could release bound material. Pretreatment of plasma with methanol, ethanol or acetonitrile did not provide a satisfactory increase in precision.

Derivatization with o-phthalaldehyde was briefly investigated but was not further pursued when the derivative was found to be very unstable.

Separation

Figure 2 illustrates a typical chromatogram for a plasma sample spiked with WR 2721 at the 5 µg/mL level. Note that the

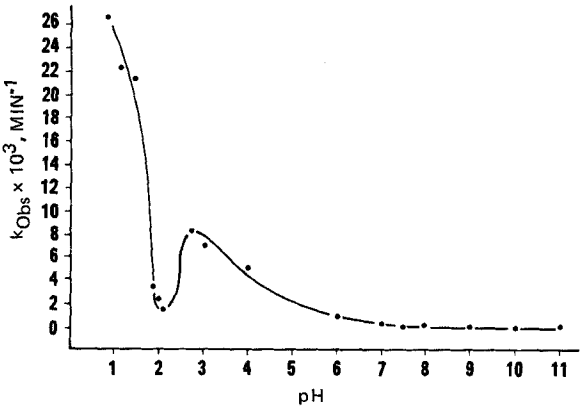

Figure 1. pH-Rate Profile for Hydrolysis of WR 2721 at 37°C

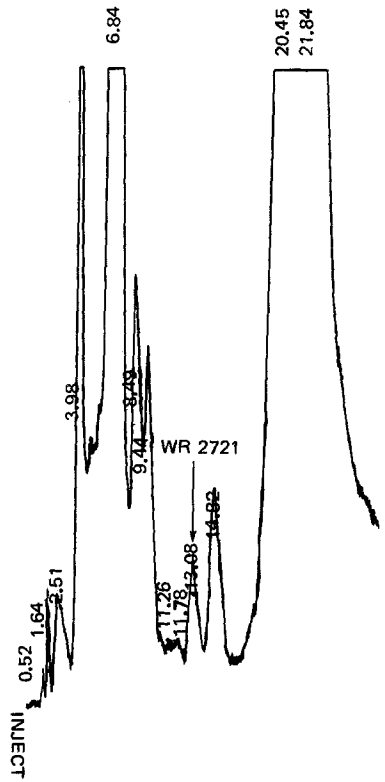

Figure 2. Chromatogram of Beagle Plasma Containing WR 2721 (5 μg/mL)

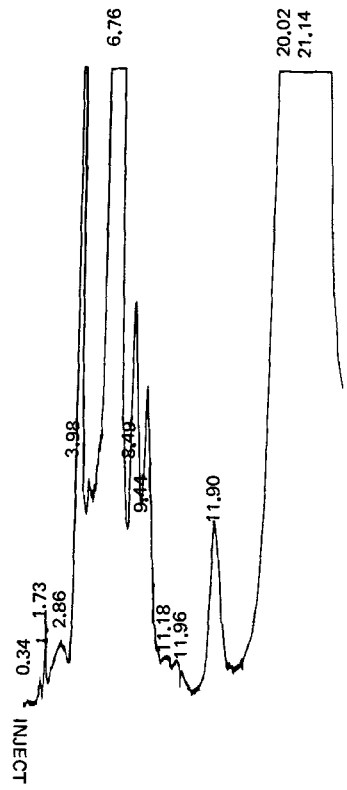

Figure 3. Chromatogram of Beagle Plasma (Blank)

peak of interest was well separated from those peaks due to endogenous materials, thought to be amino acids. A chromatogram of a plasma blank is shown in Figure 3.

The separation was performed under isocratic conditions to increase sample throughput and increase precision. As a result, less polar materials (including WR 1065 and WR 33278) were not eluted and, when allowed to remain, seriously degraded resolution. Flushing the column with methanol/water (70:30) at the end of each analysis day restored column resolution and increased its lifetime.

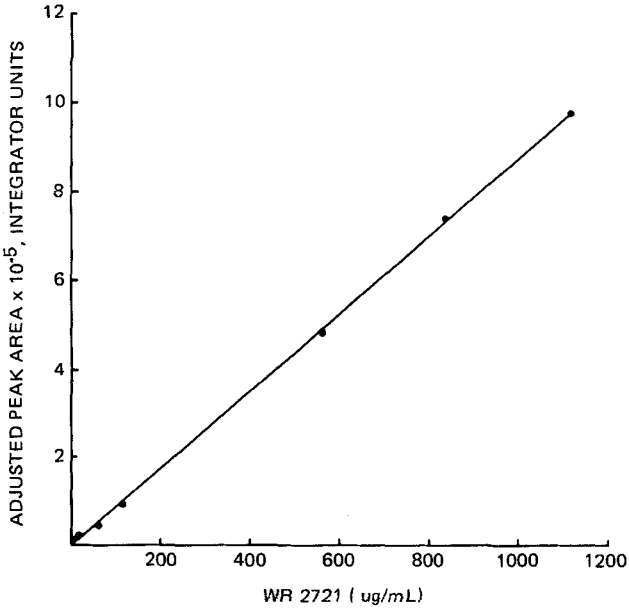

Figure 4. Standard Curve. Samples of beagle plasma spiked with WR 2721 and internal standard were derivatized and chromatographed. Points were corrected for recovery of internal standard.

Internal Standard

As can be seen in Figure 2, the complex chromatogram of derivatized plasma did not offer much hope of finding a suitable internal standard, i.e., one which would have similar behavior toward hydrolysis during sample storage and workup, and elute in a reasonable time in an unobstructed region of the chromatogram. A number of homologs of WR 2721, several other phosphorothioates and a number of amino acids were screened without success. Therefore, radiolabeled WR 2721 was added to plasma samples immediately after they were drawn and the column effluent was collected and counted to determine recovery. Excellent precision and accuracy values were obtained over the approximate range of 2 to 1100 μg/mL using this procedure.

TABLE 1
Precision of WR 2721 Analytical Method

WR 2721 Concentration, g/mL	Number of Replicates Analyzed, n	RSD (%)
1110	5	9.1
112	5	5.3
11.9	5	5.6
6.3	5	5.1
3.0	5	7.8
	Average =	6.6

TABLE 2
Accuracy of WR 2721 Analytical Method

Spike Level, g/mL	Measured Level, g/mL	% Deviation (D)
1.89	1.92	1.6
8.55	8.75	2.3
34.1	33.1	-3.0
78.6	75.9	-3.4
112	107	4.5
390	430	10.2
779	742	-4.8
1110	1050	-5.4

$$\text{Average Deviation} = \frac{|D|}{n} = 4.4$$

Linearity

Figure 4 depicts the relationship between the drug plasma levels and the peak area for derivatized WR 2721. Linear regression of peak area versus concentration gave a coefficient of determination (R^2) of 0.9969, with slope of 930 and intercept of -7684.

Precision and Accuracy

Precision of the method over the entire working range was determined by the analysis of replicate spiked samples. In

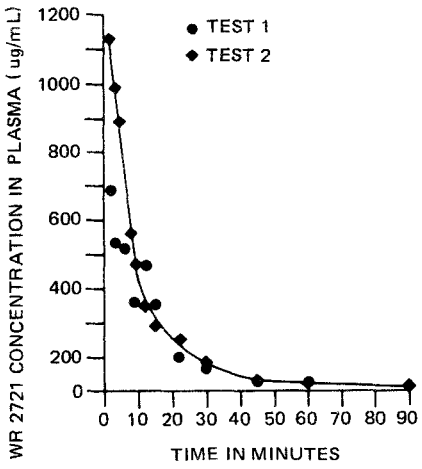

Figure 5. WR 2721 Concentration in Beagle Plasma as a Function of Post-Infusion Time

Table 1 the concentration, number of replicates analyzed and the relative standard deviation (RSD) for each data set is presented. Average RSD for the method was 6.6%.

Accuracy of the method for plasma concentrations ranging from 1.89 to 1110 g/mL was determined by the analysis of blind spiked plasma samples. Average deviation for eight determinations was 4.4%. Spike levels and measured levels are presented in Table 2.

BIOLOGICAL APPLICATION

Plasma levels of WR 2721 were monitored after two intravenous dosings of a beagle dog. As shown in Figure 5, the experiments gave essentially the same profile; from an initial level of approximately 1000 μg/mL, the drug level approached the assay sensitivity (~2 μg/mL) within 90 minutes. Samples taken after longer periods did not decay to the zero level and work is in progress to increase sensitivity and to determine whether a low level (<1 μg/mL) interference was present.

SUMMARY

A procedure for the analysis of WR 2721 contained in plasma has been developed which requires only a fast derivatization reaction and HPLC separation. The method has been tested in pilot dosing experiments with a beagle dog in which drug levels have been measured from 2 to 1100 μg/mL. Work is in progress to extend the sensitivity of the method and to allow the use of a nonradiolabeled internal standard.

ACKNOWLEDGEMENT

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