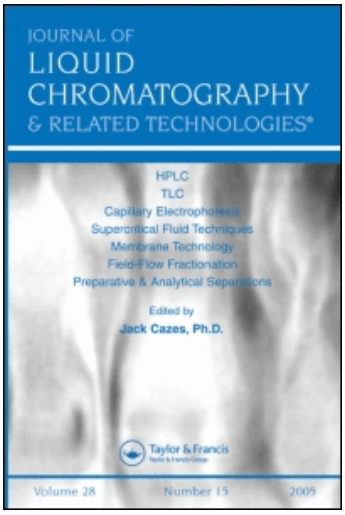

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A Liquid Chromatographic Electrochemical Assay for S-2-(3-Aminopropylamino) Ethylphosphorothioate (WR2721) in Human Plasma

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A LIQUID CHROMATOGRAPHIC ELECTROCHEMICAL ASSAY FOR S-2-(3-AMINOPROPYLAMINO)ETHYLPHOSPHOROTHIOATE (WR2721) IN HUMAN PLASMA

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ABSTRACT

A liquid chromatographic electrochemical method for the determination of the radioprotective drug WR2721 in human plasma has been developed. This method includes the use of a Hg/Au electrochemical detector for the direct measurement of WR2721 concentration. An analog of WR2721, S-3-(4-aminobutylamino) propylphosphorothioate (WR80855) is the internal standard. The retention times for WR2721 and WR80855 are approximately 4.5 and 9 minutes, respectively. WR1065, the free sulfhydryl metabolite of WR2721, is retained on the column under the described chromatographic conditions and therefore does not interefere with the determination of the parent drug. With modification of the mobile phase WR1065 is eluted from the column at a retention time of approximately 20 minutes. This method has good linearity, precision and accuracy, and is free from interference from endogenous plasma substances. Preliminary results showing the applicability of this method to human pharmacokinetic studies and to investigating the enzymatic hydrolysis of WR2721 are presented.

INTRODUCTION

A number of studies in animals have shown that WR2721 provides significant protection of normal tissues from radiation injury (1,2). Recent observations suggest that this experimental

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drug will also protect normal tissues from alkylating agent toxicity (3). On the other hand, the radiosensitivity of a number of solid animal tumors is not altered by the administration of WR2721. The recent observation of facilitated uptake of WR2721 by normal cells compared to passive absorption by solid tumors has been proposed to account for its differential protective effect (4). The protective effect of WR2721 is presumed to result from the appearance, at a very rapid rate, of its dephosphorylated free sulfhydryl metabolite, WR1065, within cells (5). WR2721 is now in clinical trials in the United States and Japan (6,7,8).

In order to pursue pharmacological and pharmacokinetic studies of WR2721 in man an assay for WR2721 is required which is reliable, fast, not subject to interference from endogenous substances and that could readily be adapted to the investigation of metabolites. We describe here our HPLC method. The direct electrochemical detection of WR2721 in the column effluent is based on the finding that it is oxidized at a potential of +0.15 volts at the surface of a mercury/gold amalgam electrode.

MATERIALS AND METHODS

Apparatus

A Bioanalytical Systems LC-154 liquid chromatograph including a dual piston pump operated at 3,000 psi and a single mercury/gold detector was used as recently described by Allison and Shoup (9). 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 5μ (4.6 x 250 mm). The mobile phase was continuously purged with nitrogen to remove dissolved oxygen.

Chemicals

S-2-(3-aminopropylamino)ethylphosphorothioate (WR2721), S-2-(3-aminopropylamino)ethanethiol (WR1065) and S-(3-aminobutyl-

amino)propylphosphorothioate (WR80855) 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 sodium octyl sulfate was from Eastman Kodak Co. (Rochester, NY). Acid phosphatase isoenzyme 2A prepared from human seminal fluid was a gift from Dr. Norman Yang, isoenzyme 5 prepared from human spleen was a gift from Dr. Bill Lam. Human liver alkaline phosphatase was a gift from Dr. Claude Petitclerq and the calf intestine enzyme (Type XXX-TA) was obtained from Sigma Chemical Co. (St. Louis, MO). All other reagents used were of the highest analytical grade available.

Sample Preparation and Chromatography

Plasma specimens were prepared, at 4 °C, immediately from blood drawn into EDTA vacutainer tubes which had been stored in an ice bath. To a 1 mL aliquot of each plasma sample was added 0.1 mL of a 1 mmol/L aqueous solution of the internal standard WR80855 and the samples were then stored at -70 °C until they were analyzed. Just prior to analysis the plasma specimens were thawed and maintained at 0 °C in an ice bath. In order to remove plasma proteins prior to liquid chromatographic analysis an equal volume aliquot of ice-cold acetonitrile was added to an aliquot of each thawed plasma specimen. After mixing, the samples were spun in a refrigerated centrifuge at 4 °C. Twenty microliter aliquots of the supernatants were injected onto a Biophase 5μ octadecylsilane column (250 x 4.6 mm) that was maintained at 25 °C with a constant temperature jacket. Elution of WR2721 and the internal standard WR80855 was achieved isocratically using an aqueous mobile phase containing 0.1 mol/L monochloroacetic acid and 1.5 mmol/L sodium octylsulfate, pH 3.0, at a flow rate of 2.0 mL/min.

Pharmacokinetic Study

In order to evaluate the application of the HPLC method for determining WR2721 in human plasma, a patient was given a single 300 mg dose (3.4 mg/kg) as a 10 second intravenous bolus. Blood samples were obtained prior to and after completion of the infusion of WR2721 at 2, 2.5, 3, 4, 8, 12, 20, 30, 45 minutes and 1, 2, 4, 8 and 24 hours. The WR2721 plasma concentration versus time data obtained on the patient were analyzed using the MK MODEL II PLUS version of the Extended Least Squares Nonlinear Regression Program, ELSNLR (10).

Acid and Alkaline Phosphatase Assays

Acid phosphatase catalytic assay conditions are essentially those described by Kachmar and Moss (11). The reaction mixture contained in final concentrations, p-nitrophenyl phosphate, 5 mmol/L; sodium citrate, 100 mmol/L, pH 5.0; and 0.1 mL of enzyme solution in a total volume of 1.0 mL. Incubation was for 30 minutes at 37 °C. The reaction was stopped with 0.1N NaOH and absorbance measured at 405 nm. In testing for the possible hydrolysis of WR2721 by acid phosphatase, the latter was substituted for p-nitrophenyl phosphate at a final concentration of 0.2 mmol/L and the incubation at 37 °C conducted for 30 minutes. A control reaction mixture consisted of all constituents except the acid phosphatase. After 30 minutes the reaction tubes were placed in ice and then 20 µL aliquots were injected onto the HPLC column for WR2721 analysis.

Using p-nitrophenyl phosphate as substrate, alkaline phosphatase activity was measured. The reaction mixture contained in final concentrations: p-nitrophenylphosphate, 16 mmol/L; tris (hydroxymethyl)aminomethane, 50 mmol/L, at pH values of 7.4, 8.0, 8.6, 9.2 and 10.0; magnesium acetate, 2 mmol/L; and 0.02 mL of enzyme in a total reaction mixture volume of 1.0 mL. Incubation was for 15 minutes at 37 °C. Absorbance was measured at 405 nm

after the addition of 0.1N NaOH. In order to determine the rate of hydrolysis of WR2721 by alkaline phosphatase the latter substrate was substituted for p-nitrophenylphosphate at a final concentration of 0.2 mmol/L and the incubation at 37 °C conducted for 15 minutes. Control reaction mixtures consisted of all constituents except for alkaline phosphatase at each of the above pH values. At the completion of the incubation period the reaction tubes were placed in ice and the 20 µL aliquots were injected onto the HPLC column for WR2721 analysis. In a separate chromatographic run these reaction mixtures were analyzed for WR1065.

RESULTS AND DISCUSSION

Detection

A differential pulse voltammogram of a solution of WR2721 in the HPLC mobile phase, (an aqueous solution of 0.1 mol/L monochloroacetic acid and 1.5 mmol/L sodium octylsulfate, pH 3.0), is displayed in Figure 1. From this experimental data it is clear that WR2721 would be oxidized on the surface of a Hg/Au electrode set at an operating potential of +0.15 volts. Thus the HPLC column effluent was monitored with a single Hg/Au working electrode at an operating potential of +0.15 volts. A typical chromatogram showing detector response versus elution time for WR2721 and the internal standard WR80855 is displayed in Figure 2.

It is important to emphasize the fact that in establishing experimental conditions for this HPLC assay for WR2721 considerable precautions were taken to minimize hydrolysis. Since WR2721 is reported to undergo nonenzymatic hydrolysis with increasing rates as pH is lowered, conditions in this method minimize the possibility of any loss of WR2721 due to hydrolysis: plasma specimens (standards and patient specimens) are stored at -70 °C until analyzed; use of a neutral polar organic solvent (acetoni-

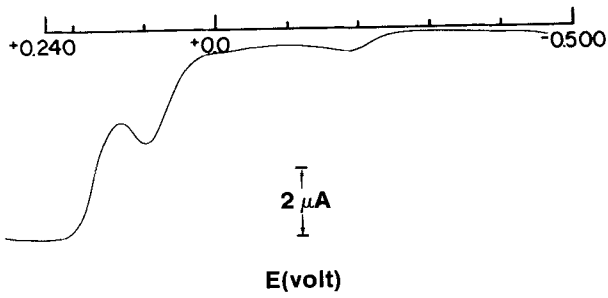

FIGURE 1. A differential pulse voltammogram of 4.5 mmol/L WR2721 in an aqueous solution of 0.1 mol/L monochloroacetic acid and 1.5 mmol/L sodium octylsulfate, pH 3, using a Hg/Au electrode.

trile) instead of an acid, such as perchloric acid, for protein precipitation; a relatively short chromatography time and, therefore, short time in the pH 3.0 mobile phase. Swynnerton, et al. (12) have measured the rate of nonenzymatic hydrolysis of WR2721 as a function of pH at 37 °C. Using a value for k_{obs} of 0.007 min^{-1} and the standard kinetic equation for a first order reaction, it is predicted that in 5 minutes at 37 °C, pH 3.0, 3.44% of WR2721 would be hydrolyzed. Since the retention time for WR2721 is less than 5 minutes and the operating temperature of the HPLC column is 25 °C our experimental conditions should not produce significant losses of WR2721 due to hydrolysis.

Linearity, Recovery, Precision and Accuracy

As shown in Figure 3 the response of the Hg/Au detector was linear over the WR2721 concentration range of 1 to 1000 μ mol/L (R^2 = 0.998). The absolute recovery of WR2721 was determined using ^{14}C-WR2721 added to normal plasma (Table 1). 98.4% (68,688/69,774 x 100) of ^{14}C-WR2721 added to normal plasma was recovered in the acetonitrile supernatant. Eighty-nine percent (61,112/68,688 x 100) of the labelled WR2721 in the supernatant aliquot applied to the HPLC column was recovered (a total of sixty 0.4 mL fractions were collected and counted).

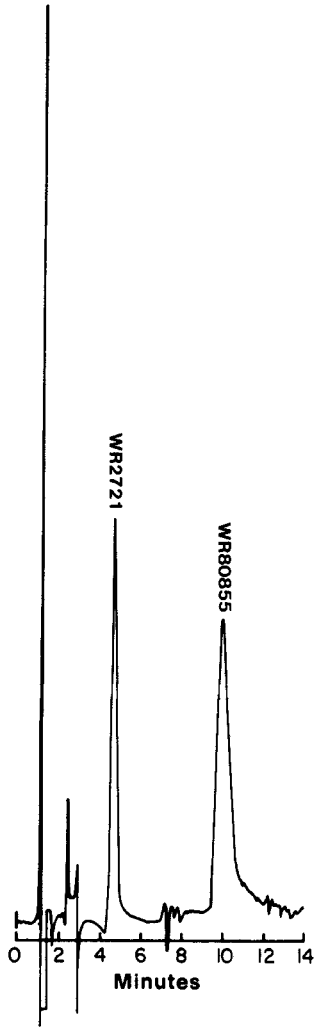

FIGURE 2. Chromatogram of 500 μmol/L WR2721 and 100 μmol/L WR80855 in normal human plasma. Sensitivity is 50 nA full scale through 7.3 min at which point it was changed to 10 nA. The acetonitrile extraction step and other experimental details are described in Materials and Methods.

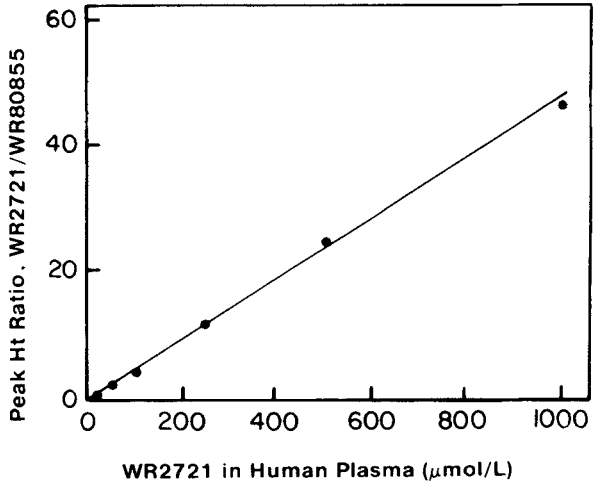

FIGURE 3. The average of duplicate peak height ratios of WR2721 to WR80855 over the range of WR2721 concentrations in normal human plasma of 1 to 1000 μmol/L are plotted against WR2721 concentration.

TABLE 1

Recovery of ^{14}C-WR2721[a] from Spiked Normal Human Plasma[b]

	Counts per min. per 0.02 mL
Plasma containing ^{14}C-WR2721 and unlabelled WR2721 (100 μmol/L)	69,774
Acetonitrile supernatant[c]	68,688
Total counts recovered from chromatograph[c]	61,112
Total counts recovered in WR2721[c] peak	53,388

[a]S-2-(3-aminopropylamino)ethyl-1,2-^{14}C-phosphorothioic acid.

[b]Human plasma from a healthy drug-free subject was collected in EDTA vacutainer tubes. 0.02 mL of a freshly prepared ice-cold solution, in 10 mmol/L phosphate buffer, pH 7.4, of 2.5 mmol/L WR2721 and 1.87 X 10^6 DPM ^{14}C-WR2721 was added to 0.48 mL of the ice-cold plasma pool.

[c]Values corrected for dilution of plasma by an equal volume of acetonitrile.

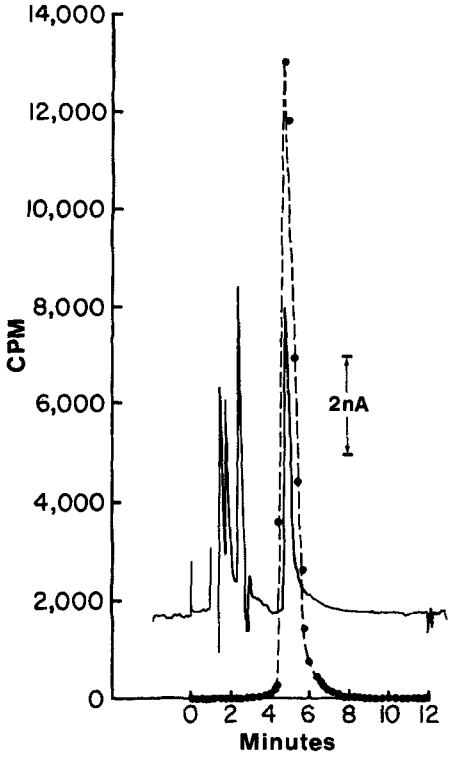

FIGURE 4. Chromatogram of 100 μmol/L WR2721 in human plasma to which ^{14}C-WR2721 had been added. A plot of the ^{14}C cpm for each of sixty collected effluent fractions is displayed as a function of time after the sample was injected. Other experimental details are described in Table 1.

Examination of the chromatogram obtained for this experiment (Figure 4) shows that no radioactivity peak other than that corresponding to the WR2721 chromatographic peak could be detected. Of the total ^{14}C counts recovered from the column, 87% (53,388/61,112 x 100) was recovered in the WR2721 peak. The net recovery of WR2721 carried through all of the steps in the procedure is therefore 76.5% (53,388/69,774). These recovery data compare favorably to those obtained by Swynnerton, et al. (12)

TABLE 2

Determination of the Precision and Accuracy of the HPLC Method

Spiked-in WR2721 Conc. µmol/L	Mean Measured Conc.[a]	Precision (SD)CV[b]	Percent Deviation (D)[c]
2	1.74	(0.11)6.5%	-13.0
5	5.3	(0.36)6.5%	6.0
40	35.8	(1.22)3.4%	-10.5
200	211	(7.38)3.5%	5.5
800	785	(10.1)1.2%	-1.9

Average % deviation = 7.4 (The average of the sum of the absolute values of D)

[a]The mean measured concentrations obtained with our HPLC method for each of four plasma samples at each WR2721 concentration using individually spiked specimens. The internal standard was 100 µmol/L WR80855.

[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.

with their recently described fluorescamine derivatization HPLC method.

With WR80855 as an internal standard we obtained the precision and accuracy data summarized in Table 2. Using aliquots of a normal human plasma pool to which WR2721 was added to final concentrations ranging from 2 to 800 µmol/L we obtained an average coefficient of variation of 4.4% and an average deviation from the spiked-in concentration value of 7.4%.

Chromatography of S-2-(3-Aminopropylamino)ethanethiol (WR1065)

WR1065, the free sulfhydryl metabolite of WR2721, does not interfere with the HPLC assay for WR2721. In control experiments we tested for the possible appearance of this compound in our

chromatograms. WR1065 did not elute from the column during 20 minutes after injection of 20 μL of 0.1 mmol/L and 4 mmol/L solutions of this compound in 10 mmol/L tris(hydroxymethyl)aminomethane, pH 7.4, or of 20 μ L of 0.1 mmol/L and 4 mmol/L of WR1065 in 10 mmol/L phosphate buffer, pH 7.4.

By modifying the mobile phase used for WR2721 chromatography to include 30% methanol it was possible to elute WR1065 from the Biophase ODS 5μ column in 20 minutes at a flow rate of 1 mL/min (Figure 5).

Human Pharmacokinetic Study

A pharmacokinetic study was performed on a patient treated with 300 mg (3.4 mg/kg) of WR2721 administered as a 10 second intravenous bolus dose. Blood samples were obtained prior to and after completion of WR2721 administration at 2, 2.5, 3, 4, 8, 12, 20, 30, 45 minutes and 1, 2, 4, 8 and 24 hours. A semilog plot of WR2721 concentration versus time is displayed in Figure 6 and a typical chromatogram of this patients' plasma compared to the pre-dose plasma is shown in Figure 7. Examination of the data in Figure 6 shows that the majority of the drug was cleared from plasma within about 5 minutes. Consistent with this conclusion is the very short distribution half-life, T1/2 , of 0.84 minute and the rapid clearance from the central compartment, Cl, of 0.977 L/hr/kg calculated from the data points with the extended least squares nonlinear regression (ELSNLR) program (10).

Enzymatic Hydrolysis of WR2721

It has been shown in experiments with mice that within 15 minutes after administration of an intravenous dose of WR2721 the principal metabolite in most tissues is WR1065 (5). Previous studies with mammalian tissues have raised the possibility that WR2721, or phosphorothioates with similar structure such as cysteamine S-phosphate, are hydrolyzed by either acid phosphatase

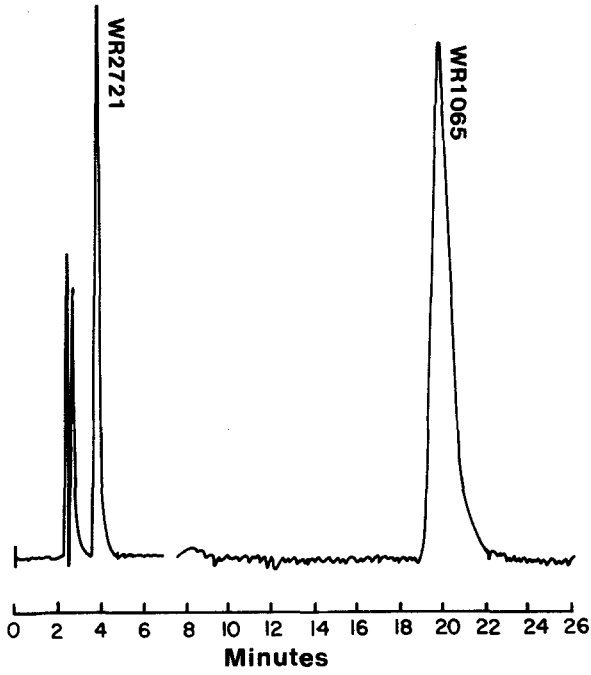

FIGURE 5. Chromatogram of an aqueous solution of 200 μmol/L WR2721 and 200 μmol/L WR1065 in 2.7 mmol/L EDTA and 10 mmol/L tris(hydroxymethyl)aminomethane, pH 7.4. The mobile phase is 30% methanol, by volume, in water. Monochloroacetic acid and sodium octylsulfate, pH 3.0, are in the mobile phase at final concentrations of 0.1 mol/L and 1.5 mmol/L, respectively. The mobile phase flow rate is 1.0 mL/min. Sensitivity was changed from 500 nA full scale to 100 nA full scale at 7 min.

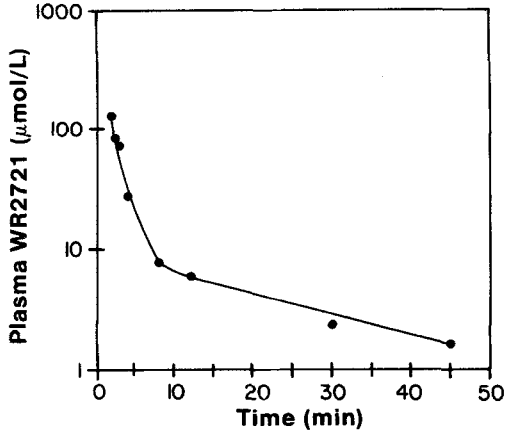

FIGURE 6. A semilog plot of WR2721 plasma concentration versus time. Each point is the average of duplicate determinations. The line fitting the points is the least squares best fit line determined with the ELSNLR program (10).

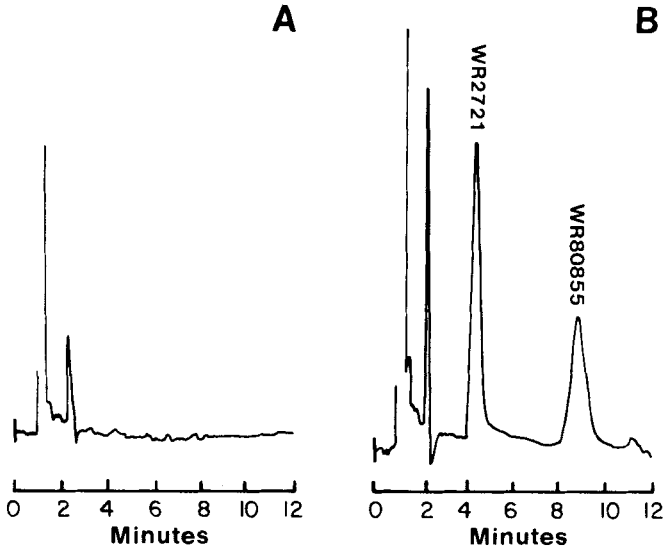

FIGURE 7. Chromatograms of acetonitrile-plasma supernatants prepared from A, plasma obtained from a patient immediately before a 300 mg intravenous bolus dose and B, plasma obtained from the patient two minutes after the dose. The sensitivity is 50 nA full scale.

(13,14) or alkaline phosphatase (15,16,17) to produce the corresponding free sulfhydryl metabolite. As shown in Table 3, WR2721 is not hydrolyzed by either human acid phosphatase isoenzyme 2A prepared from prostatic fluid as previously described (18) or isoenzyme 5 from human spleen (19). On the other hand, human liver alkaline phosphatase, prepared as described by Daigle (20) and calf intestine alkaline phosphatase do catalyze the hydrolysis of WR2721 (Table 3). The fact that the rate of WR2721 hydrolysis was higher than that achieved with PNPP as substrate using the human liver enzyme, but lower using the calf intestine enzyme may result from kinetic differences between the alkaline phosphatase isoenzymes. More detailed kinetic studies will be required to characterize this difference.

TABLE 3

Rates of Hydrolysis of p-Nitrophenylphosphate and WR2721 by Acid and Alkaline Phosphatases

	Hydrolysis Rates[a] (nmol/min/mL reaction mixture)	
	WR2721	p-Nitrophenylphosphate
Acid Phosphatase		
I. Isoenzyme 2A[b]	0.0002	2.75
II. Isoenzyme 5[c]	0	2.07
Alkaline Phosphatase		
I. Human liver[d]	11.49	6.56
II. Calf intestine[e]	11.25	508

[a]Each activity value is the average of duplicate determinations as described in Materials and Methods.

[b]Each reaction mixture contained 2.75 mU of isoenzyme 2A acid phosphatase per mL with p-nitrophenylphosphate as substrate. In testing for possible WR2721 hydrolysis each reaction mixture contained 44,000 mU of isoenzyme 2A acid phosphatase per mL.

[c]Each reaction mixture contained 2.07 mU of isoenzyme 5 acid phosphatase per mL with p-nitrophenylphosphate as substrate. In testing for possible WR2721 hydrolysis each reaction mixture contained 83 mU of isoenzyme 5 acid phosphatase per mL.

[d]Each mL of reaction mixture contained 6.6 mU of human liver alkaline phosphatase.

[e]Each mL of reaction mixture contained 508 mU of calf intestine alkaline phosphatase. All alkaline phosphatase reaction mixtures were incubated at pH 8.6.

The rate of WR2721 hydrolysis as a function of pH using human liver alkaline phosphatase is shown in Figure 8. The maximal rate of hydrolysis catalyzed by human liver alkaline phosphatase was obtained at pH 8.6. In contrast the optimal pH for the synthetic substrate, p-nitrophenylphosphate, is much higher than 8.6 (Figure 8). The highest activity was at pH 10 (the highest pH value tested). Previous studies have obtained pH optima, with p-nitrophenylphosphate as substrate, of 10.2 for alkaline phosphatase extracted from human liver cells grown in tissue culture

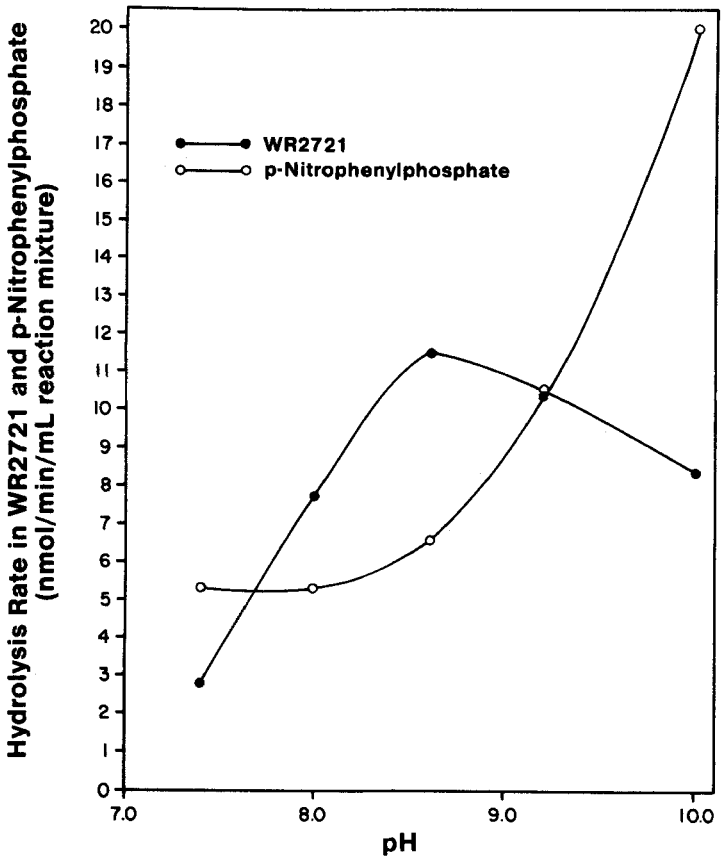

FIGURE 8. A plot of the rates of hydrolysis of WR2721 and p-nitrophenylphosphate by human liver alkaline phosphatase as a function of pH. Each ml of reaction mixture contained 6.6 mU of enzyme.

(21) and 10.4 for alkaline phosphatase purified from human liver obtained at autopsy (22). Our finding of different pH optima for these two alkaline phosphatase substrates is consistent with previous observations of the dependence of the pH optimum for alkaline phosphatase on both the chemical nature of the substrate as well as on the substrate concentration (23).

CONCLUSIONS

The HPLC electrochemical detection method reported here for the measurement of WR2721 has the following advantages: a) it is rapid, since each chromatographic cycle is 15 minutes; b) it is a direct method which does not require additional derivatization steps; c) it is both precise and accurate; d) it eliminates plasma proteins prior to chromatography using a neutral polar organic solvent; e) neither the free sulfhydryl metabolite of WR2721, WR1065, nor endogenous substances from patients' plasma co-elute with either WR2721 or WR80855; f) it is readily applicable to the study of WR2721 pharmacokinetics in humans and to the investigation of its metabolism by mammalian alkaline phosphatases.

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