약제학회지 (1987), 제17권 제2호
J. Kor. Pharm. Sci., Vol. 17, No. 2, 55~60 (1987)

An Improved HPLC Assay Using Hg/Au Electrochemical Detector for S-2-(3-aminopropylamino) ethylphosphorothioate and S-2-(3-methylaminopropylamino) ethylphosphorothioate in Human Plasma

Kun Han and Emil T. Lin*
College of Pharmacy, Chungbuk National University
**School of Pharmacy, University of California, San Francisco*
(Received April 17, 1987)

사람혈장중 S-2-(3-Aminopropylamino) ethylphosphorothioate 및 S-2-(3-Methylaminopropylamino) ethylphosphorothioate의 수은/금 전기화학검출기를 이용한 고속액체크로마토그라프법에 의한 분석

한 건·Emil T. Lin*
충북대학교 약학대학·*캘리포니아대학 샌프란시스코 약학과
(1987년 4 월 17일 접수)

WR 2721 (S-2-(3-aminopropylaminoethylphosphorothioate) is a radioprotective drug that is now undergoing clinical trials in the United States and Japan. a liquid chromatographic electrochemical method for the determination of WR 2721 an WR 3689 [S-2-(3-methylaminopropylamino)ethylphorothioate] in human plasma was developed in this study. This method includes the use of a Hg/Au electrochemical detector and a cyano column for the direct measurement of WR 2721 and WR 3689 in plasma. An analog of WR 2721, WR 149846·was used as an internal standard. WR 2721 and WR 3689 could be well separated from the solvent front, with a mobile phase of acetonitrile-water (20:80), 0.1M acetic acid and 1.2 mM sodium octane sulfonate. This method was shown to be precise. Both intra-day and inter-day results were within 10% CV. Also, sample preparation was fairly simple. Since WR 2721 and WR 3689 were unstable at room temperature, it was essential to use an automatic sample processor with a refrigerator, especially for carrying out routine analyses.

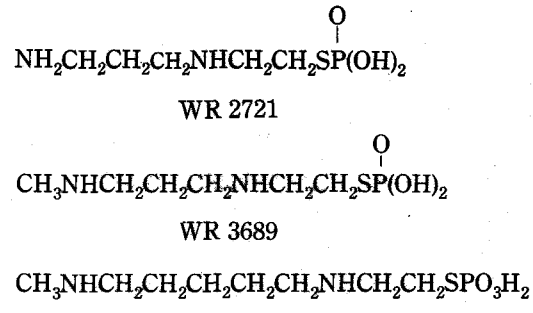
WR 2721 [S-2-(3-aminopropylamino) ethylphosphorothioate] is an expermental drug that provides significant radioprotection to many normal tissues but provides little or no protection to many experimental tumors.[1,2]

WR 3689 [S-2-(3-methylaminopropylamino) ethylphosphorothioate] is quite similar in structure to WR 2721, differing only in that WR 3689 has a terminal methyl group on the amine end. Several reports have shown that WR 2721 provides significant protection against the toxic effects of the chemotherapeutic drugs, cisplatin and cyclophosphamide.[3,4]

In order to study the pharmacology and pharmacokinetics of WR 2721 and WR 3689 in man, an assay is required that is reliable, fast and not subject to interference from endogenous substances. Of the two assays for WR 2721 that we considered, one involved derivatization with fluorescamine followed by fluorescence (F) detec-

tion[5] and the other used electrochemical (EC) detection on a mercury-gold cell with no derivatization.[6] Both EC and F detectors are selective and have comparable detection limits, but we decided to develop an HPLC assay for WR 2721 and WR 3689 and to use EC detection to avoid the difficulty in derivatizing a secondary amine. In any case, derivatization is not suited to unstable compounds such as WR 2721 and WR 3689.

Here we report a sample preparation method that is simple and which assures the stability of WR 2721 and WR 3689 in plasma samples. This method includes the use of a Hg/Au electrochemical detector at the selective potential of +0.15 volts and a cyano column for the direct measurement of WR 2721 concentration. An analog of WR 2721, WR 149846, was used as an internal standard.



WR 2721

WR 3689

WR 149846

EXPERIMENTAL

Reagents

The WR compounds (2721, 3689 and 149846) were supplied by the US Army (Walter Reed Army Medical Center). These compounds were kept in a minus 20°C freezer from the time of arrival until use. The ion-pairing reagent, sodium octane sulfonate (SOS), was obtained from Regis Chemical Co. (Morton Grove, IL). Acetonitrile was from Fisher Scientific (Santa Clara, CA). Acetic acid was purchased from Mallinckrodt (Los Angeles, CA). Water was purified with a Nanopure Apparatus (Barnstead, Waters Assoc., Milford, MA). The mobile phase was thoroughly mixed, then degassed by filtration through a ground-glass filter (Millipore Corp., Bedford, MA).

Instrumentation

A Bioanalytical Systems (BAS) LC-4B amperometric detector (W. Lafayette, IN) in the oxidation mode was used with a mercury-gold electrode. An HP 3392A Reporting Integrator (Hewlett-Packard, Santa Clara, CA) and a dual-piston pump (Model 100A, Beckman Instruments, Palo Alto, CA) were used. The pump was run at a flow rate of 1.2 m*l*/min. The system was equipped with a WISP 710B automatic sample processor (Waters Assoc., Milford, MA) with refrigerator. The Altex Ultrasphere CN column (5μ, 4.6 × 2500 mm) came from Beckman.

Sample Preparation

A stock solution of WR 3689 (2.431 mg/m*l*) and WR 2721 (2.264 mg/m*l*) were diluted with water to make high and low concentration standard working solutions (243.1 μg/m*l* and 24.31 μg/m*l* for WR 3689, 226.4 μg/m*l* and 22.64 μg/m*l* for WR 2721). A stock solution of WR 149846 (116.1 mg/m*l*) was diluted with water to make the internal standard working solution (46.4 μg/m*l*). Plasma samples (0.2 m*l*) were spiked with the standard working solutions of WR 3689 to give final concentrations of 0.122, 0.243, 0.608, 1.22, 2.43, 4.86, 9.72, 18.2 and 36.5 μg/m*l* and also another plasma samples (0.2 m*l*) were spiked with the standard working solutions of WR 2721 to give final concentrations of 0.113, 0.226, 0.566, 1.13, 2.26, 4.53, 9.06, 17.0 and 34.0 μg/m*l*. After 25 μl of the internal standard working solution was added, the samples were vortexed for 30s. WR compound and the internal standard were added to the plasma samples in an ice bath. Following the addition of 0.4 m*l* of acetonitrile, the samples were vortexed for 1 min and centrifuged at 3000g for 10 min. The supernatant of each sample was transferred to an automatic sample (WISP) vial and a 10 μl aliquot of each sample under 2°C was injected onto HPLC column.

System Conditions

The mobile phase consisted of 20% acetonitrile, 0.1 M acetic acid and 1.2 mM SOS. The flow rate was 1.2 *ml*/min. The run time totaled 14 min. The detector oxidation potential during system operation was set at +0.150 volts.

RESULTS AND DISCUSSION

Mobile Phase

By using a CN column instead of the ODS-C-18 column, we obtained chromatograms with better peak shape and improved the sensitivity of the method. The best potential for WR 2721 was determined to be +0.150V (Fig. 1) Because a CN column was used for determination of WR 2721, the experimental conditions for the mobile phase were thoroughly checked. A series of studies were conducted i.e. changing percent acetonitrile, changing sodium octane sulfonate (SOS) concentration, and changing the acetic acid concentration in order to obtain a reasonable retention time and peak shape. Unlike most other chemicals, the retention times of thiophosphates, such as WR 2721, increases with an increase in the percent acetonitrile. It appeared 20% acetonitrile, 2 mM SOS, and 0.3 M acetic acetic acid was the best mobile phase for determining WR 2721. However, under these conditions, the retention time of WR 2721 was too short and the drug peak could not be separated from the solvent front. The mobile phase was, therefore, modified to a composition of 20% acetonitrile, 1.2 mM SOS, and 0.1 M acetic acid to get a retention time of approximately 7.4 min for WR 2721 and 8.5 min for WR 3689. Fig. 2 illustrates typical chromatograms for human plasma samples spiked with WR 2721 and WR 3689.

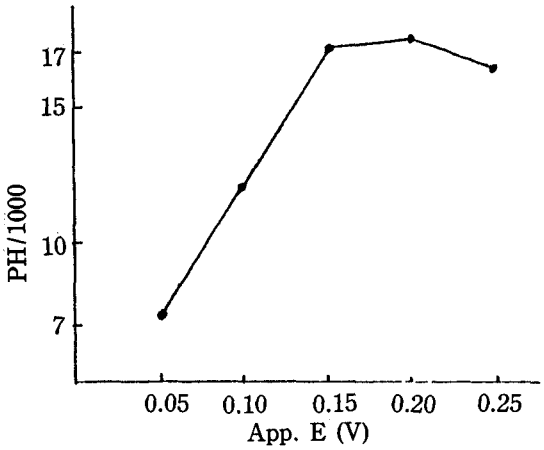


Figure 1—Peak height (PH) of WR 2721 versus applied potential (App. E).

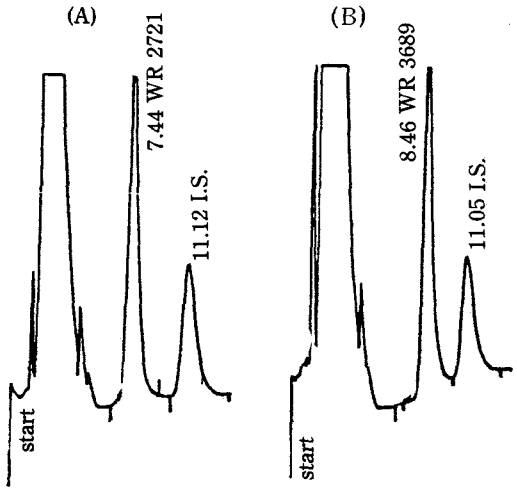


Figure 2—Representative chromatograms showing separation of (A) WR 2721 and WR 149846, (B) WR 3689 and WR 149846 in human plasma. The spiked concentrations for chromatograms (A) and (B) were 9.06 and 9.72 $\mu g/ml$, respectively.

Internal Standard

A number of homologue 2721 were screened to find an internal standard for the assay, and WR 249846 was found to separate well. Therefore, WR 149846 was chosen as the internal standard.

Linearity

Table Ia reveals the relationship between the drug plasma level and the peak height ratio of WR 2721/WR 149846 for drug concentrations from 0 to 34.0 $\mu g/ml$. Linear regression analysis of the peak height ratio (PHR) of drug to internal standard versus concentration gave a coefficient of determination(r^2) of 0.9995 with slope of 0.3122 and intercept of 0.0555. Linear regression analysis of PHR's versus spiked concentrations gave a best fit line with a y-intercept of minus 0.0131, a

Table Ia—*Plasma WR 2721 Standard Curve Peak Height Ratios*

Concentration (μg/ml)	Peak height ratio[1)]	Calculated conc.[2)] (μg/ml)
0	0	
0.113	0.041	0.176
0.226	0.066	0.263
0.566	0.150	0.556
1.13	0.322	1.16
2.26	0.603	2.14
4.53	1.264	4.44
9.06	2.611	9.13
17.0	5.299	17.2
34.0	10.578	34.1

[1)]Regression equation for peak height ratios:
[2)]From 0 to 9.056 μg/ml, $y = 0.287x - 0.0096$ ($r^2 = 0.9985$); From 0 to 33.960 μg/ml, $y = 0.3122x - 0.0555$ ($r^2 = 0.9995$)

Table Ib—*Plasma WR 3689 Standard Curve Peak Height Ratios*

Concentration (μg/ml)	Peak height ratio[1)]	Calculated conc.[2)] (μg/ml)
0	0	
0.122	0.036	0.154
0.243	0.065	0.244
0.608	0.181	0.607
1.22	0.351	1.14
2.43	0.742	2.36
4.86	1.573	4.96
9.72	3.088	9.75
18.2	5.893	19.2
36.5	10.977	35.9

[1)]Regression equation for peak height ratios:
[2)]From 0 to 9.724 μg/ml, $y = 0.3198x - 0.0131$ ($r^2 = 0.9997$); From 0 to 36.5 μg/ml, $y = 0.3046x - 0.0398$ ($r^2 = 0.9993$)

slope of 0.3198, a correlation coefficient(r^2) of 0.9997 for samples ranging from 0 to 9.72 μg/ml of WR 3689. In the range from 0 to 36.5 μg/ml of WR 3689, Linear regression gave a best bit line with a *y*-intercept of minus 0.0398 a slope of 0.3046, and a r^2 of 0.9993 (Table Ib).

Precision Study

Precision of the method was determined by the analysis of replicate spiked samples (n=6) as shown in Tables II and III. Coefficients of variation (CV) ranged from 2.16 to 5.29% with an average of 3.58% for the four concentration levels in the intra-day precision analysis of WR 2721. CV's ranged from 4.38 to 6.45% with an average of 5.48% for the four levels in the inter-day precision analyses of WR 2721. Inter-day precision analyses of WR 3689 resulted in CV of 9.12% or less. Intra-day precision analyses of WR 3689 resulted in CV of 4.73% or less.

Stability Studies

Because WR compounds are very unstable at room temperature, sample preparation and handl-

Table II—*Intra-Day Precision of WR 2721 and WR 3689 Plasma Assay*

Compound	Spiked conc., μg/ml	Day 1	2	3	4	5	6	Mean	SD	%CV
WR 2721	17.0 (High)	17.8	17.2	17.6	17.6	17.4	18.4	17.7	0.413	2.33
	9.06 (Medium)	8.69	9.36	9.38	9.48	8.49	9.23	9.11	0.412	4.52
	2.26 (Low)	2.16	2.05	2.09	2.07	2.09	2.03	2.08	0.045	2.16
	0.566 (X-Low)	0.544	0.500	0.518	0.535	0.474	0.488	0.510	0.027	5.29
WR 3689	36.5 (High)	38.1	35.9	35.8	37.0	35.5	36.5	36.1	0.539	1.40
	18.2 (Medium)	18.5	19.5	20.0	17.4	19.0	19.0	18.9	0.894	4.73
	4.86 (Low)	5.11	4.83	4.84	4.80	4.70	4.87	4.88	0.136	2.80
	0.608 (X-Low)	0.630	0.627	0.614	0.612	0.596	0.566	0.608	0.024	3.95

Table III—*Inter-Day Precision of WR 2721 and WR 3689 Plasma Assay.*

Compound	Spiked conc., μg/ml		Day						Mean	SD	%CV
			1	2	3	4	5	6			
WR 2721	17.0	(High)	18.2	19.2	17.5	16.7	17.5	16.2	17.6	1.07	6.08
	9.06	(Medium)	9.08	10.1	9.46	9.16	9.19	8.99	9.33	0.409	4.38
	2.26	(Low)	1.99	2.03	2.28	2.18	2.14	2.08	2.12	0.106	5.00
	0.566	(X-Low)	0.563	0.536	0.613	0.641	0.572	0.594	0.587	0.068	6.45
WR 3689	36.5	(High)	32.4	33.4	34.6	33.6	34.2	33.3	33.6	0.765	2.28
	18.2	(Medium)	20.4	21.8	22.9	21.3	20.3	20.3	21.2	1.05	4.95
	4.86	(Low)	5.01	4.79	4.61	4.80	4.68	4.76	4.78	0.136	2.85
	0.608	(X-Low)	0.667	0.592	0.621	0.611	0.516	0.546	0.592	0.054	9.12

Table IV— *Intra-Day Precision of WR 2721*[1] *Assay at Room Temperature.*

Spiked conc., μg/ml	Set order						Mean	SD	%CV
	1	2	3	4	5	6			
5.56	6.209	5.081	4.732	4.732	4.476	2.819	4.675	1.096	23.44
2.78	3.003	2.337	2.585	1.692	1.522	1.432	2.095	0.641	30.59
1.39	1.313	1.036	1.050	0.795	0.924	0.629	0.958	0.235	24.55
0.348	0.503	0.373	0.409	0.391	—	0.272	0.390	0.083	21.22

[1]Samples were run in set order, each set 1.5 hr apart.

ing required special care. During sample preparation (30 min–1 hr) and waiting for injection, signifi cant degradation of WR 2721 can be seen and the standard curve becomes skewed when samples are not refrigerated. Table IV shows some intra-day data, where all the samples were run over a 24 h period at room temperature. The % CV's were more than two times greater than the CV's for samples that were kept cold. Therefore, it is essential to use a refrigerated WISP for sample processing, especially for carrying out routine analyses. Degradation half-life ($T_{1/2}$) versus various pH shows that WR 2721 is most stable at plasma pH 7.4 (Fig. 3). So it was not necessary to adjust plasma pH. And the degradation peak comes out with retention time 34.1 min in acidic plasma (pH 3.9) and 31.9 min in basic plasma (pH 11.0). Also we could detect WR 1065, the metabolite of WR 2721 with retention time of 86 min under these conditions, however the assay would have to be developed.

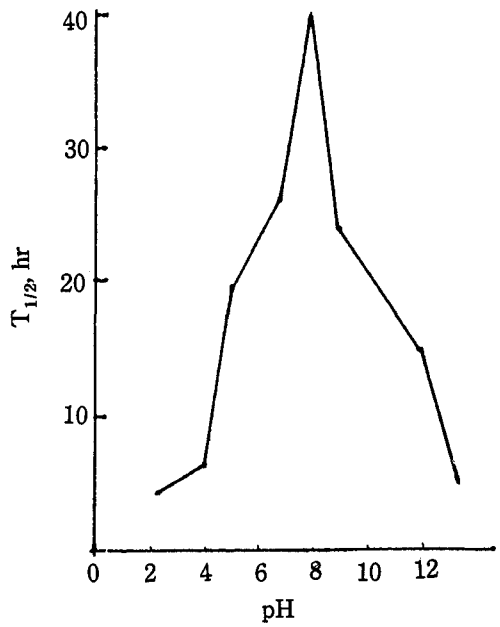


Figure 3—Degradation half-life of WR 2721 at various plasma pH and room temperature.

CONCLUSIONS

By using a liquid chromatographic electrochemical method with Hg/Au electrode, we have developed the analytical methods for WR 2721 and WR 3689 in human plasma. This method has been shown to be precise. Both intra-day and inter-day results were within 10% CV. Also, sample preparation was fairly simple and by using a refrigerated WISP, we could reduce problems with stability even though WR 2721 was most stable at pH 7.4. WR 1065, the metabolite of WR 2721, could also be detected with retention time of 86 min under these conditions.

REFERENCES

1) J.M. Yuhas and J.B. Storer, Differential chemoprotection of normal and malignant tissues, *J. Nat'l. Cancer Inst.*, **42**, 331 (1969)
2) D.E. Davidson, M.M. Grenan and T.R. Sweeney, Biological characteristics of some improved radioprotectors in *Radiation Sensitizers, Their Use in the Clinical Management of Cancer, Brady,* L.W., ed., Masson Publications, USA, 1980, p. 309-320
3) J.M. Yuhas and F. Culo, Selective inhibition of the nephrotoxicity of cisplatinum without altering its antitumor effectiveness, *Cancer Treat. Rep.*, **64**, 57 (1980)
4) J.M. Yuhas, J.M. Spellman and S.W. Jordan, Treatment of tumors with the combination of WR 2721 and cis-dichlorodiammine platinum or cyclophosphamide, *Br. J. Cancer,* **42**, 574 (1980)
5) D.J. Mangold, *et al.,* HPLC Assay for S-2-(3-aminopropylamino) ethylphosphorothioate (WR 2721) in plasma, SW *Research Institute Study Report 2* (1983)
6) L.M. Show, *et al.,* A liquid chromatographic assay for S-2-(3-aminopropylamino) ethyl phosphorothioate (WR 2721) in human plasma, *J. Liq. Chromatogr.*, **7**(12), 2447 (1984)