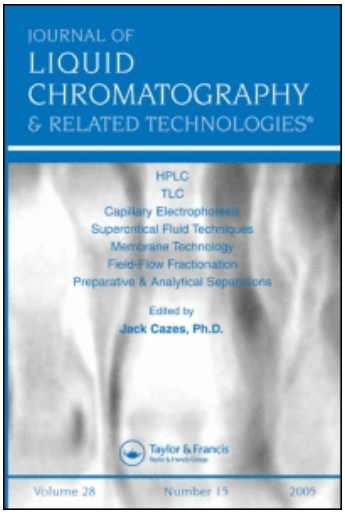

Journal of Liquid Chromatography & Related Technologies

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

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To cite this Article Bacolod, Maria D. , Lopez-Anaya, Arturo , Kamath, Burde and Kishore, Vimal(1996) 'Studies of the Stability of a Substituted Phosphorothioate Using an Improved High Performance Liquid Chromatography Assay Coupled with Electrochemical Detection', Journal of Liquid Chromatography & Related Technologies, 19: 8, 1277 — 1289
To link to this Article: DOI: 10.1080/10826079608006318
URL: http://dx.doi.org/10.1080/10826079608006318

J. LIQ. CHROM. & REL. TECHNOL., 19(8), 1277–1289 (1996)

STUDIES OF THE STABILITY OF A SUBSTITUTED PHOSPHOROTHIOATE USING AN IMPROVED HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ASSAY COUPLED WITH ELECTROCHEMICAL DETECTION

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ABSTRACT

A sensitive, reproducible and rapid HPLC assay was developed for preformulation and stability studies of a potential radioprotector, S-2-(3-methylaminopropylamino) ethyl-phosphorothioate, WR-3689. This method is based on the elution of WR3689 by high performance ion-pair reverse phase liquid

chromatography using an electrochemical detector with an oxidation potential set at + 0.19 v. The detector response was found to be linear over the concentration range of 0.02 - 20 μg/mL with an absolute limit of quantitation of 170 pg. The coefficient of variation ranged from 0.75 - 6.52% for the interday reproducibility. This HPLC assay was employed to determine the stability of WR-3689 at various pH (2, 4, 7, 10 and 12) and temperatures (22 °C, 35 °C, 43 °C and 100 °C). Results of the stability studies indicated that WR-3689 is more stable in basic than in acidic aqueous solutions. For example, the half-life of WR-3689 at 45 °C was 13.8 days at pH 12 as compared to 12.7 h at pH 4. With regard to the effect of temperature, the half-life of this compound increased from 19.2 h at 43 °C to 41.8 days at 22 °C. In conclusion, the excellent reproducibility and stability indicating capacity of this assay makes it a useful method for formulation studies. The high sensitivity and specificity of this assay make it useful for pharmacokinetic studies.

INTRODUCTION

Chemical radiation protectors are receiving considerable attention as cancer treatment agents. Several sulfhydryl (-SH) containing compounds, particularly aminothiols and phosphorothioates have been found to exhibit chemical radioprotection through three general mechanisms, i.e., radical scavenging, hydrogen atom donation and oxygen depletion.[1-4] Thus far, WR-2721, S-2-(3-aminopropylamino) ethylphosphorothioate is the most widely studied phosphorothioate compound.[5-6] It is currently being studied in clinical trials involving patients undergoing radiotherapy and chemotherapy treatment.[7,8] However, at present, WR-3689 (S-2-(3-methylaminopropylamino) ethylphosphorothioate) is gaining attention due to the recent findings that it is a better radioprotector than WR-2721 when given orally.[3,9,10] In addition, WR-3689 was found to be a more potent radioprotector than WR-2721 at a low dose that produced no behavioral side effects.[4] Hence, collaborative drug development studies involving WR-3689 are being conducted.

During drug development, it is important to assess the stability of drugs of interest. One of the major drawbacks in studying the stability of certain drugs is the scarcity of simple and efficient analytical methods for such purposes. The fact that among phosphorothioate compounds, WR-2721 and its metabolites are the most widely studied compound is due to the availability of various assays. Among these are high performance liquid chromatography

$CH_3NHCH_2CH_2CH_2NHCH_2CH_2SPO_3H_2$

A

$CH_3NHCH_2CH_2CH_2CH_2CH_2NHCH_2CH_2SPO_3H_2$

B

Figure 1. Chemical Formula of (A) WR-3689 and (B) WR149846.

(HPLC) assays involving the use of fluorescence[11-13] and electrochemical detection.[14-17] For WR-3689, a compound that differs from WR-2721 by one methyl (-CH3) unit (Fig.1), only one method for its measurement in plasma has been published.[18] This method, however, has few shortcomings which when improved will result in a very powerful HPLC assay for the analysis of WR-3689 and related compounds. As will be discussed later, our method provides several advantages without affecting or sacrificing the high sensitivity and efficiency which was exhibited by the previously published method. Indeed, the development of this simple, efficient and fast HPLC assay for WR-3689 is important not only for stability studies but also for preformulation as well as pharmacokinetic studies.

MATERIALS AND METHODS

Instrumentation

The chromatographic system used in this study consisted of a Waters Model 590 programmable solvent delivery module and Waters Intelligent Sample Processor (WISP) Model 712 (Milford, MA). A Bioanalytical Systems (BAS) Model LC-4C electrochemical detector (W. Lafayette, IN) equipped with a Hg/Au electrode was used. This assembly was connected to a Maxima 820 chromatography station.

Reagents

Reagent grade chloroacetic acid and 1-sodium heptane sulfonate (SHS) and sodium octyl sulfate were purchased from Sigma Chemical Company (St. Louis, MO). HPLC grade acetonitrile was from Fisher (Fair Lawn, NJ), sodium hydroxide was from Mallinckrodt, Inc. (St. Louis, MO), glacial acetic acid

and concentrated hydrochloric acid were from J.T. Baker (Phillipsburg, NJ). Both WR-3689 and WR-149846 were supplied by the U.S. Army (Walter Reed Army Medical Center, Bethesda, MD).

Chromatographic Conditions

The mobile phase consisted of 5 mM SHS and 5% acetonitrile in 0.1 M chloroacetic acid at pH 3.0. The column used was a C_{18} (Spheri-5 ODS, 5 μm, 250 x 4.6 mm I.D.) from Bioanalytical Systems (West Lafayette, IN). The flow rate was 1.2 mL/min and the detector (Hg, Au electrode) oxidation potential was set at +0.19 v. The injection volume was 20 μL.

Sample Preparation

For each test conducted, a freshly prepared aqueous stock solutions of WR-3689 (2.5 mg/mL) and WR-149846 (1.0 mg/mL) were prepared. Dilution of these aqueous stock solutions were made in order to prepare the desired standard solutions. In the case of the internal standard (WR-149846), a stock solution of 1.0 mg/mL was prepared.

RESULTS AND DISCUSSION

The degradation of WR-3689 in aqueous solution was followed by using ion-pair reverse phase chromatography coupled to amperometric detection (Hg/Au). This type of detection offers high selectivity and sensitivity, hence, requires a minimum amount of sample (μL level) to give a quantifiable response even at very low concentrations of the analyte (μg/mL level). The detector reaction is quite indirect and is based on the oxidation of mercury in the presence of certain species such as thiols. The sulfhydryl (-SH) group of WR-3689 forms a complex with the mercury (Hg). It is actually the sulfhydryl-Hg complex which undergoes an oxidation reaction at a given potential.[19] This is best described by the following reaction:

$$2RSH + Hg \text{ --------- } Hg(SR)^2 + 2H^+ + 2e$$

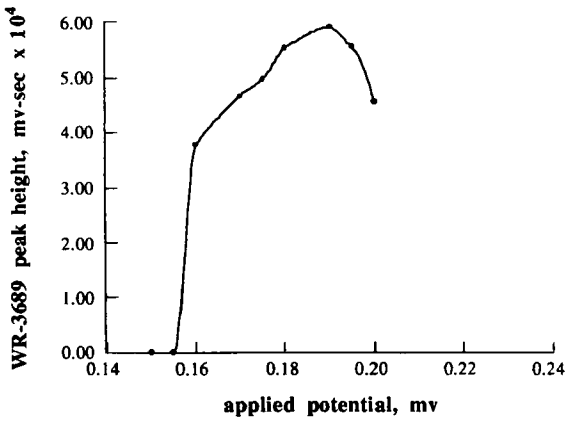

Figure 2. Plot of the measured peak heights of WR-3689 at various applied potentials. An electrochemical detector with Hg/Au working electrode set at 0.1μA.

In order to obtain the highest response, the best applied potential was determined by measuring the peak heights of WR-3689 (25 μg/mL) at various oxidation potentials under the conditions described by Han and Lin.[18] The results obtained illustrate that + 0.19 v is the most appropriate applied oxidation potential for WR-3689 detection (Fig. 2).

As mentioned earlier, for the quantitative determination of WR-3689 by HPLC, only one published method exists.[18] Although this method proved to be very sensitive and efficient, there are two major shortcomings which are very apparent: a.) the relatively long total analysis time and, b.) the use of a relatively high organic solvent concentration (20 % acetonitrile).

Because WR compounds are known to be unstable at room temperature, making the analysis time as short as possible is very essential especially when analyzing several samples. On the other hand, the use of a lower concentration of organic solvent in the mobile phase is more cost effective and reduces the problems usually associated with organic waste disposal. Most importantly, it is necessary to reduce the amount of organic solvent in the mobile phase since its presence in high concentration may lead to the leaching of the mercury from the working electrode, hence, decreasing the sensitivity of the assay over time.

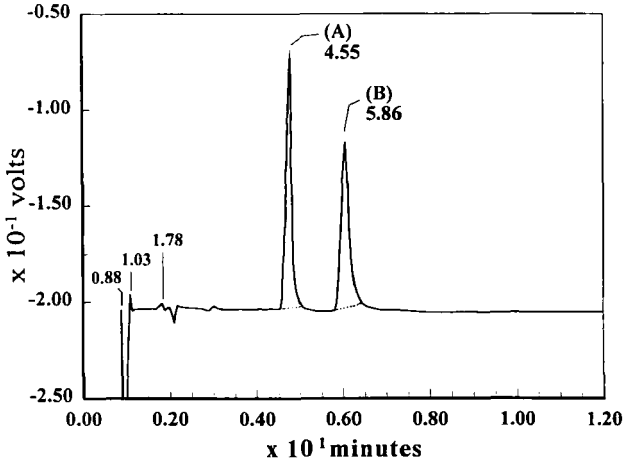

Figure 3. A typical chromatogram showing the elution and separation of the analyte WR-3689 (A) and the internal standard W R- 149846 (B).

To improve the shortcomings of the only available HPLC assay for the quantitative determination of WR-3689, several chromatographic conditions were investigated. Among the conditions evaluated, the ion-pair reverse phase chromatography using an octadecyl column (C_{18}) and a mobile phase consisting of 5.0 mM sodium heptane sulfonate and *5%* acetonitrile in 0.1 M chloroacetic acid at pH 3.0 was found to be the best in terms of selectivity, peak shape and analysis time (Fig 3).

The above-described HPLC assay offers several advantages over the previously published method. With this method, shorter analysis time was obtained as indicated by the lowering of the retention times of both WR-3689 and its internal standard, WR-149846, by about 47 % (i.e., from 8.5 min to 4.5 min and from 11.1 min to about 5.8 min for WR-3689 and WR-149846, respectively). As expected, the use of the weaker ion pairing agent, sodium heptane sulfonate, instead of the commonly used stronger ion pairing agent, sodium octane sulfonate, resulted in an adequate resolution at lower retention times for both WR-3689 and WR-149846, hence leading to a shorter analysis time.

Under these conditions, efficient separation was obtained even with 75 % reduction in acetonitrile concentration. As discussed earlier, the use of a lower acetonitrile concentration provides several benefits.

Table 1

Interday and Intraday Reproducibility of the HPLC Assay

	Conc. μg/mL	Coefficient of Variation (%)
Interday[a]	12.5	0.75 - 6.14
	125	2.17 - 6.52
Intraday[b]	12.5	2.91
	125	2.16

[a] Results were taken from five determinations each for five days.
[b] Results were taken from five determinations on the same day.

Reproducibility of the HPLC Assay

The inter- and intraday reproducibility of the HPLC assay was evaluated by chromatographically analyzing 12.5 μg/mL and 125.0 μg/mL WR-3689 solutions. The intraday reproducibility was measured by calculating the coefficient of variations between the peak height ratios obtained from five determinations within the same day. For interday reproducibility measurements, the correlation coefficients between peak height ratios of five replicates for five days were calculated. The results of this study are summarized in Table 1. The interday coefficient of variation ranged from 0.75-6.14% and 2.17-6.52% for 12.5 μg/mL and 125 μg/mL WR-3689, respectively. For intraday reproducibility, the coefficient of variation was found to be 2.91% and 2.16% for the low and high concentrations of WR-3689, respectively.

Linearity

To 5 μL of WR-3689 solutions (0.4, 4, 40, 200 and 400 μg/mL in water), 5.0 μL of the internal standard and 100μL of deionized water were added. The peak height ratios of the WR-3689/internal standard were plotted against the

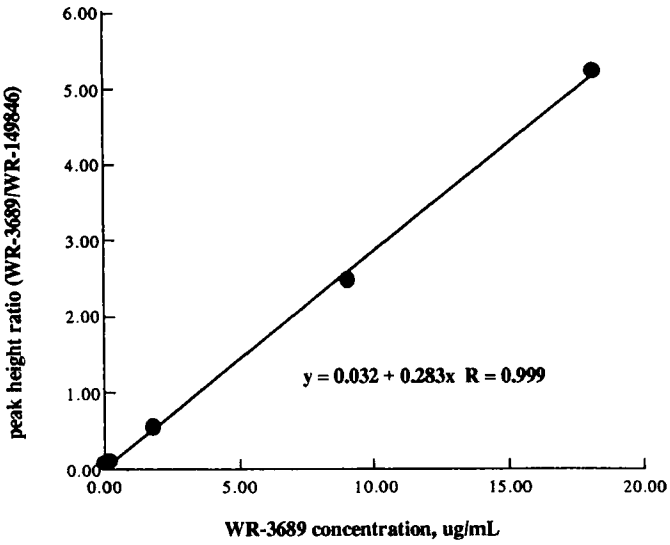

Figure 4. Linearity of the HPLC assay at various WR-3689 concentrations spiked with a constant concentration of WR-149846 (internal standard). The results were obtained from the means of five replicates at each concentration.

Table 2

Summary of the Observed Degradation Rate Constants (k) and Half-Lives ($t_{1/2}$) of WR-3689 at Various pH Conditions at 45 °C

pH	k (day^{-1})	$t_{1/2}$
2	49.6	28.8 min
4	1.30	12.7 h
10	0.70	23.5 h
12	0.05	13.83 days

corresponding WR-3689 concentration (Fig. 4). Linear regression analysis resulted in a best fit line with a y-intercept of 0.032 and a slope of 0.283 and a correlation coefficient (r^2) of 0.999, hence, indicating an excellent linearity over a concentration range of about 0.02 -20 μg/mL.

Absolute Limit of Quantitation

To determine the sensitivity of the assay, the absolute limit of quantitation was measured. Based on the 3 signal/noise ratio (3 S/V), the limit of quantitation was found to be 170 pg.

Stability Studies of WR-3689

Stability studies are necessary in order to determine the conditions and length of time in which the drug is effective without getting converted into inactive or toxic products. In this report, the stability of WR-3689 was studied under different temperature and pH conditions.

Effect of pH

The effect of pH on the stability of WR-3689 at a constant temperature was investigated. Initially, attempts were made to determine the effects of pH at 100 °C. Under this condition, WR-3689 was found to completely degrade (as indicated by the complete disappearance of the WR-3689 peak) after 5-10 minutes at pH 2 and 4 and after 2-3 hours at pH 10 and 12. Since the decomposition rates of WR-3689 at 100 °C were too fast to follow, solutions at various pH were kept at a lower temperature, i.e., 45 °C.

The plots of the logarithm of percent WR-3689 concentration *versus* time display the dependence of WR-3689 stability on pH at a constant temperature. The stability of WR-3689 was found to increase with pH (Fig. 5 and Table 2). Table 2 shows that the half-life ($t_{1/2}$) of WR-3689 at 45 °C increased from 28.8 min at pH 2 to 13.8 days at pH 12. On the other hand, the degradation rate constants (k) decreased with increasing pH, i.e., from 49.6/day at pH 2 to 0.05/day at pH 12. This observation is consistent with the earlier studies on the stability of a very similar phosphorothiote, WR-2721.[11,20] This drug was found to exhibit an increasing non-enzymatic hydrolysis with decreasing pH. At 37 °C, WR-2721 was found to have half lives of 0.4 h to 8.0 h over the pH range of 1-3.

Effect of temperature

In order to evaluate the influence of temperature on WR-3689 stability, solutions at pH 7 were kept at 22 °C, 35 °C and 43 °C. Table 3 summarizes the observed degradation rate constants as well as the half-lives of WR-3689 at

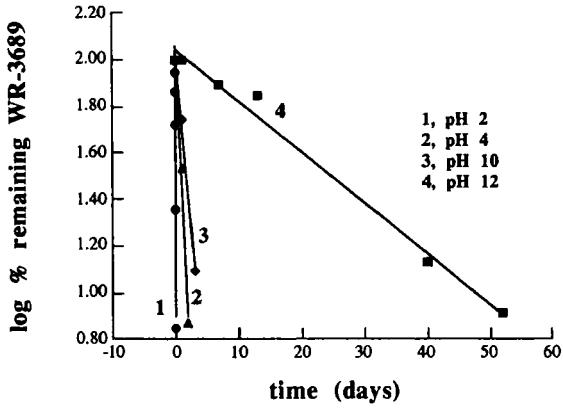

Figure 5. Effect of pH on the stability of WR-3689 at 45°C.

Table 3

Summary of the Observed Degradation Rate Constants (k) and Half-Lives ($t_{1/2}$) of WR-3689 at Various Temperatures at pH 7

Temperature (°C)	k (day^{-1})	$t_{1/2}$
22	0.02	41.75 days
35	0.13	5.41 days
43	0.86	19. 2 hours

various temperatures. The degradation rate constant (k) of WR-3689 was found to increase with increasing temperature at a constant pH. It increased from 0.02/day at 22 °C to 0.86/day at 43 °C. Conversely, the half life decreased with increasing temperature, i.e., from 41.8 h at 22 °C to 19.2 h at 43 °C. These results were found to be parallel with those obtained with WR-2721. For example, the $t_{1/2}$ values for the hydrolysis of WR-2721 were 107 h and 87 min at 0°C and 25 °C, respectively.

An Arrhenius plot of the log of the observed degradation rate constant as a function of the reciprocal of the absolute temperature was linear with a correlation coefficient of 0.998 (Fig. 6). The activation energy (Ea) for the

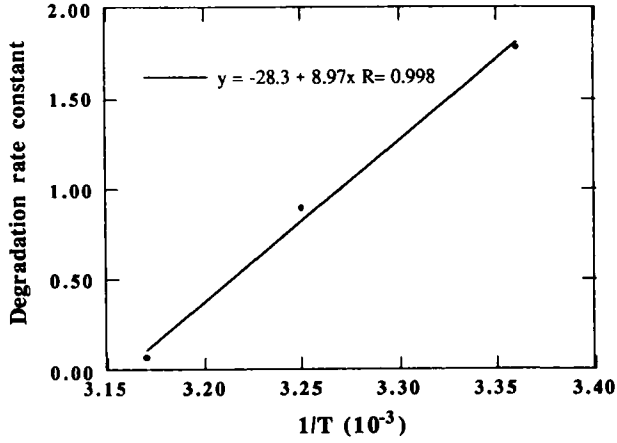

Figure 6. Arrhenius plot of the degradation of WR-3689.

decomposition of WR-3689 at pH 7.0 over the temperature range studied (22 °C- 43 °C) was 41 kcal/mol. The fairly low E_a obtained indicates that WR-3689 is fairly stable in aqueous solution at pH 7.0.

The finding that WR-3689 degrades by about 1% in one day at 25 °C indicates that the loss of WR-3689 is insignificant during the experimental process where it is exposed for at least five minutes at 25 °C at pH 7.0.

CONCLUSION

The HPLC assay that we have developed proved to be very simple, efficient, reproducible and capable of analyzing a large number of samples in a short period of time. This method offers several advantages over the currently available HPLC assay for WR-3689. This assay was employed for the stability studies of WR-3689 in aqueous solutions at various pH and temperature. Results of the study showed that the stability of WR-3689 is greatly influenced by both temperature and pH.

This HPLC assay is being validated for the stability studies of WR-3689 in biological fluids. In future, it is anticipated that this assay will become a very useful tool in the pharmacokinetic studies of WR-3689 in biological fluids.

ACKNOWLEDGMENT

This study was supported by a grant to the Tulane/Xavier Center for Bioenvironmental Research by the Defense Nuclear Agency.

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Received June 13, 1995
Accepted July 13, 1995
Manuscript 3897