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Chromatographic and mass spectral analysis of the radioprotector and chemoprotector *S*-3-(3-methylaminopropylamino)propanethiol (WR-151326) and its symmetrical disulfide (WR-25595501)

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Abstract

Metabolically active forms of the radioprotective and chemoprotective drug *S*-3-(3-methylaminopropylamino)propylphosphorothioic acid (WR-151327) are *S*-3-(3-methylaminopropylamino)propanethiol (WR-151326) and its symmetrical disulfide (WR-25595501). This paper describes applications of sensitive and specific procedures such as capillary column gas chromatography with flame ionization detection, electron impact mass spectrometry and liquid chromatography with electrochemical detection for structural characterization and analysis of the active forms of WR-151327. These chromatographic procedures provide reproducible linear calibration graphs for a relatively wide range of concentrations of the active forms of WR-151327. The described procedures will further facilitate in vivo and in vitro investigations of chemoprotective and radioprotective properties of WR-151327 and its active metabolites.

Keywords: Radioprotector; Chemoprotector; Aminothiols; Symmetrical disulfides; Mass spectral analysis; Electrochemical detection

1. Introduction

The use of chemical agents such as cysteine for protection against acute effects of irradiation was first investigated in the late 1940s by Patt et al. [1]. In 1959, the US Army initiated an antiradiation drug development program at the Walter Reed Army Institute of Research (WRAIR) [2]. In this program, phosphorylated aminothiols, or phosphorothioates, were developed.

Recently, antimutagenic and antineoplastic properties for this class of chemical agents have been observed [3,4] and the ability of these compounds to enhance the interaction of topoisomerases with DNA has been reported [5]. The active forms of the phosphorothioates are the free

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thiol and its symmetrical disulfide [6] (see Table 1). The dephosphorylation of the phosphorothioate compounds has been shown to occur in vivo and in vitro in the presence of the enzyme alkaline phosphatase [7,8] or mineral acids[9]. In the presence of oxygen and trace metal ions, these thiols can be further oxidized to form their corresponding symmetrical disulfides [10].

WR-2721 and its dephosphorylated form WR-1065 remain the benchmark radioprotectors; however, WR-151326, the dephosphorylated form of WR-151327 appears to be equally or more effective not only against higher linear energy transfer (LET) radiation [11] but also as a combination drug against the anti-AIDS agent AZT (3′-azido-3′-deoxythymidine) [12]. AZT is known to be mutagenic in the human hepatoma cell line designated HepG2 at the HGPRT (hypoxanthine guanine phosphoribose transferase) locus. Administration of WR-151326 with AZT reduces the mutagenic effects of AZT by approximately half [12].

To understand fully the protection mechanisms and therapeutically utilize these drugs with greater effectiveness, for in vivo or in vitro experiments, a rapid, selective and sensitive procedure for their analysis must be available. To the best of the authors' knowledge, such procedures for WR-151326 and WR-25595501 have not been documented. In this paper, we describe gas chromatography (GC), liquid chromatography with electrochemical detection (LC–EC) and mass spectrometry (MS) for the analysis and structural characterization of the active metabolites of WR-151327.

Table 1
Chemical names and structures of aminothiol compounds and their derivatives

Compound	Name and structure
WR-2721	*S*-2-(3-Aminopropylamino)ethylphosphorothioic acid $H_2NCH_2CH_2CH_2NHCH_2CH_2SPO_3H_2$
WR-1065	*N*-(2-Mercaptoethyl)-1,3-diaminopropane $H_2NCH_2CH_2CH_2NHCH_2CH_2SH$
WR-33278	Symmetrical disulfide of WR-1065 $H_2N(CH_2)_3NH(CH_2)_2SS(CH_2)_2$ $NH(CH_2)_3NH_2$
WR-151327	*S*-3-(3-Methylaminopropylamino)propolyphosphorothioic acid $CH_3NH(CH_2)_3NH(CH_2)_3SPO_3H_2$
WR-151326	*S*-3-(3-Methylaminopropylamino)-propanethiol $CH_3NH(CH_2)_3NH(CH_2)_3SH$
WR-25595501	Symmetrical disulfide of WR-151326 $CH_3NH(CH_2)_3NH(CH_2)_3SS(CH_2)_3$ $NH(CH_2)_3NHCH_3$

2. Experimental

2.1. Reagents and materials

All reagents were of analytical-reagent grade. Acetonitrile, methylene chloride and methanol were obtained from Fisher Scientific (Pittsburgh, PA, USA). WR-151327 (Lot #1, USBR 110) was received from the US Bioscience Laboratory (West Conshohocken, PA, USA) and authentic samples of WR-151326 (bottle #AV58457) and WR-25595501 (bottle #24307) were received from the Walter Reed Army Research Institute (Washington, DC, USA).

2.2. Synthesis, GC and MS analysis of WR-151326

In a 100 ml, two-necked, round-bottomed flask with an argon inlet and reflux condenser, 1.62 g of recrystallized WR-151327 was placed in 10 ml of 2.5 mM HCl (60°C, pH 1.5) under a constant stream of argon. To monitor the progress of the hydrolysis reaction, 10 μl aliquots from the reaction mixture were withdrawn at 30-min intervals. Each sample was quenched at room temperature in 1.8 ml of 50 mM phosphate buffer (pH 7.0) and assayed for the presence of sulfhydryl group with 5′,5′-dithiobis(2-nitrobenzoic acid) (DTNB) using the Ellman methodology [13]. The hydrolysis reaction was allowed to proceed at 65°C until no increases in the DTNB absorption assays were observed. The reaction mixture was stirred for an additional 3 h at room temperature to assure complete hydrolysis of WR-151327, after which the pH was re-adjusted to 1.5 and the mixture was lyophilized. The lyophilate was extracted three times with 10 ml of cold, degassed methylene

chloride followed by four extractions with 10 ml of chloroform. The solid residue was subsequently dissolved in a minimal quantity (<3 ml) of cold, degassed ethanol/water (9:1, v/v) and allowed to crystallize at 4°C overnight. The crystalline product was collected (1.1 g, >67% yield).

Trimethylsilyl (TMS) ether preparation was then carried out for the aliquots from the following samples: (1) lyophilate of the reaction mixture; (2) residue from the methylene chloride extract; (3) crystalline product obtained from ethanol–water recrystallization; and (4) authentic sample of WR-151326. The TMS derivatives were prepared in hypovials by first dissolving 25 μg of the material in 30 μl of warm acetonitrile and subsequently mixing this with 30 μl of bis(trimethylsilyl)trifluoroacetamide (BSTFA). The resulting mixtures were heated at 60°C for 1 h and then allowed to equilibrate at room temperature for an additional 1 h prior to GC analysis. Each TMS preparation was subsequently analyzed on a Varian 3700 gas chromatograph (Varian Associates, Sunnyvale, CA, USA) in flame ionization detection mode using a fused-silica capillary column (25 m × 0.32 mm i.d.) coated with 5% cross-linked phenylmethylsilicone gum phase in the split mode (1:10) and using helium as the carrier gas. The column oven temperature was programmed from 90 to 170°C, 5°C min^{-1}.

MS of the above-mentioned TMS samples and their corresponding underivatized samples was carried out using a Kratos 25RFA analytical mass spectrometer (Kratos Analytical, Manchester, UK) in a direct insertion probe/electron impact (DIP/EI) MS mode at 70 eV and a source temperature of 260°C.

2.3. Synthesis, GC and MS analysis of WR-25595501

WR-25595501 was synthesized from WR-151326 using both iodine-induced and metal ion-catalyzed oxidations.

2.4. Iodine-induced oxidation

In a 25 ml round-bottomed flask, a sample of WR-151326 (0.81 g) was gradually dissolved in cold 10% NaOH. To the resulting solution, 1.1 g of iodine crystals were added over a 60 min period while the reaction mixture was stirred at 4°C. The progress of the oxidation reaction was monitored by the withdrawal of 10-μl aliquots from the reaction mixture at regular intervals. Each sample was assayed for the sulfhydryl group by the DTNB method as noted above [13]. When all of the sulfhydryl groups had been consumed, the reaction mixture was lyophilized and the residue extracted twice with 20 ml of warm (37°C) acetonitrile. The acetonitrile extracts were combined and allowed to stand overnight at room temperature to crystallize the product. Crude crystalline product was collected and recrystallized in a minimal quantity of acetonitrile/water (1:1, v/v). An approximate yield of 30% was observed after further recrystallization from methanol/water (9:1, v/v).

2.5. Metal ion-catalyzed oxidation

The freshly recrystallized WR-151326 (0.81 g) was dissolved in 4.0 ml of 2 μM copper(II) sulfate and the pH of the solution was adjusted to 9.0. The solution was then saturated with oxygen gas and allowed to stand at room temperature for 48 h or until all of the free sulfhydryls were consumed. The reaction mixture was lyophilized and the residue was washed with 5 ml of cold methanol and extracted three times with 10 ml of chloroform. The chloroform extracts were combined and the chloroform evaporated under nitrogen. The residue was then recrystallized in a minimal quantity of methanol. An approximately 60% yield of the purified product was observed. The product and its authentic sample, WR-25595501, were transformed into their corresponding TMS derivatives and analyzed on a Varian GC and the observed retention times (RT) were compared. MS analysis of the derivatized and underivatized product was also performed in the DIP/EI mode to confirm the identity of the product.

2.6. GC analysis of a mixture of WR-151326 and WR-25595501

Equimolar mixtures of WR-151326 and WR-25595501 (5, 50, 100, 250, 500 and 1000 pmol)

were treated with BSTFA and transformed into their corresponding TMS derivatives. The TMS preparations were analyzed on a GC and calibration graphs for both the compounds were determined by peak integration of the corresponding GC peak area as a function of concentration.

2.7. LC–EC analysis of WR-151326 and WR-25595501

Sample purity determination and analysis of the synthesized WR-151326 and WR-25595501 compounds were performed using LC–EC on a BAS 200A chromatograph (Bioanalytical Systems, West Lafayett, IN, USA). Analysis was performed at room temperature with a 100 × 4.6 mm i.d., 5 μm, reversed-phase SynChropak^R SCD-100 column (SynChrom, West Lafayette, IN, USA) and a dual Hg/Au amalgam electrode. The mobile phase consisted of 100 mM monochloroacetic acid (pH 2.97) and was run at a flow rate of 1 ml min^{-1}. The downstream, working electrode potential was fixed at +150 mV while the potential of the upstream, "generator" electrode, was set at −1 V. (Disulfides are reduced at the upstream electrode, generating thiols for detection downstream.) The sensitivity of the working electrode was set at 500 nA full-scale. The helium sparge exhaust vent on the LC was fitted with a short length of PEEK^R microbore tubing and was used to degas all samples for at least 5 min before injection. Standards (10 mM) were prepared in 50 mM perchloric acid from the authentic samples of WR-151326 and WR-25595501 and subsequently diluted with mobile phase to achieve the desired concentrations. Calibration graphs ranging between 39 and 2500 pmol (injected) were generated. Injections (20 μl) were made using a Rheodyne Model 7725I syringe-loading injector (Rheodyne, Cotati, CA, USA) equipped with a 100 μl sample loop. Chromatographic analysis was performed using Chrom Perfect Direct (Justice Innovations, Palo Alto, CA, USA) installed on a personal computer. The analysis of each of the synthesized compound's chromatograms for their RT and redox state as compared with those of the corresponding authentic sample was used as a qualitative measure of each synthetic compound's purity. The slope and correlation coefficients were determined by linear regression analysis.

3. Results and discussion

For proper usage and pharmacological or toxicological studies, it is often necessary to assess the drug (or its metabolites) for various reasons, including stability of the drug and the presence of impurities or decomposition products. As the first step towards the methods development for the analysis of WR-151326 and WR-25595501, it was necessary to synthesize, isolate and purify these drugs. Both these active forms are known; however, neither is commercially available. To the best of the authors' knowledge, specific protocols for their preparation also have not been published. Therefore, although the primary focus of this report is at the analysis of the drugs, some necessary background details regarding the synthetic and purification procedures for these drugs are also provided.

Synthesis of WR-151326 was accomplished by acid hydrolysis of WR-151327. The product, in turn, was oxidized to WR-25595501 under relatively mild oxidation conditions. GC and LC–EC in conjunction with DIP/EI/MS methodologies were utilized to identify, characterize the chemical structures and analyze the purity of the synthesized compounds. For the GC and MS analyses, both synthetic and authentic samples of WR-151326 and WR-25595501 were transformed into their corresponding TMS derivatives [14,15] (to decrease their polarity and increase their volatility and thermal stability). In addition, aliquots from the crude lyophilate and residue from its methylene chloride extracts were individually transformed into their corresponding TMS derivatives in order to identify all products from the acid hydrolysis of WR-151327.

Fig. 1 shows the gas chromatograms of the above-mentioned TMS preparations, in which a represents the solvent front, b the TMS derivative of phosphoric acid, c the TMS derivative of WR-151326 and d the TMS derivative of WR-25595501. The identities of b, c and d were made by comparison of their GC RTs with those of the

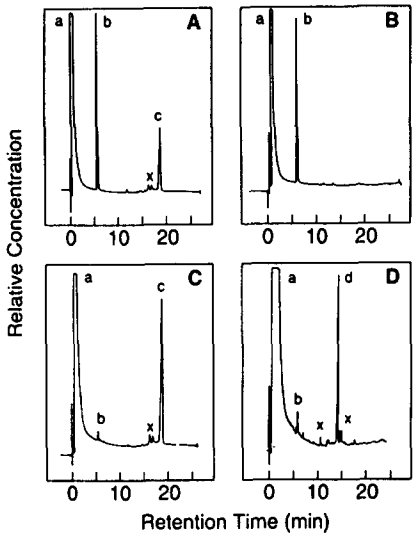

Fig. 1. Gas chromatograms of the TMS preparations: a, solvent and air; b, tri-TMS of phosphoric acid; c, tri-TMS of WR-151326; d, di-TMS of WR-2559550; x, unidentified product, perhaps partially derivatized material. (A) TMS preparation of hydrolysis products of WR-151327 prior to purification of the individual components; (B) TMS preparation of phosphoric acid after purification; (C) TMS preparation of synthetic WR-151326 after purification; (D) TMS preparation of synthetic WR-25595501 after purification.

appropriate TMS derivatives of the authentic samples and by observation of the appropriate peak enhancement when TMS derivatives of the synthesized products were mixed with those of the corresponding authentic samples and mixtures were analyzed individually. It is obvious from the GC data (Fig. 1) that acid hydrolysis of WR-151327 principally results in the formation of phosphoric acid and WR-151326 (Fig. 1A). Phosphoric acid can be isolated from the hydrolysis reaction product mixture by methylene chloride extraction (Fig. 1B). The methylene chloride-insoluble material WR-151326 can be further purified by recrystallization from ethanol–water (9:1, v/v) (Fig. 1C). WR-25595501 can be isolated from the reaction product mixture of mild oxidation of WR-151326 (Fig. 1D).

The structural identities of the synthesized compounds were confirmed by MS analysis of both their underivatized and the TMS derivatives in the DIP/EI mode. Fig. 2 shows the fragmentation pattern of the TMS derivative of WR-151326. The molecular ion, M^+, is observed at m/z 378. Other ions of significant interest are at m/z 363, 170, 143, 116 and 102, which are attributed respectively to $(M^+-{}^{\bullet}CH_3)$, $[CH_3-N(TMS)-CH_2-CH=CH-NH=CH_2]^+$, $[CH_3-NH(TMS)-CH_2-CH=CH_2]^+$, $[CH_3-N(TMS)-CH_3]^+$ and $[CH_3-NH-(TMS)]^+$. The ion at [15] m/z 73 is $[Si-(CH_3)_3]^+$. Based on the presence of the M^+ and other characteristic fragment ions, the structure of this TMS derivative was confirmed to be a tri-TMS derivative of WR-151326.

The mass spectral fragmentation pattern of the underivatized WR-151326 was consistent with the chemical structure of the compound (spectrum not shown). The observed M^+ fragmentation pattern (ion and ion relative intensity, %) was as follows; m/z at 162 (10%), 147 (90%), 131 (10%), 103 (10%) and 93 (10%).

The mass spectral identity of the TMS preparation of phosphoric acid was confirmed to be a tri-TMS derivative of phosphoric acid. The confirmation was made principally by detection of the molecular ion, M^+, at m/z 314, and its daughter ion $(M^+-{}^{\bullet}CH_3)$ at m/z 299 (spectrum not shown).

The TMS preparation of WR-25595501 under the present protocol forms the di-TMS derivative. This was confirmed by MS analysis of the TMS derivative (Fig. 3). The spectrum shows the molecular ion M^+ at m/z 466, $(M^+-{}^{\bullet}CH_3)$ at m/z 451 and $[CH_3-N(TMS)-CH_3]^+$ at m/z 116. The presence of low-intensity ions at m/z 314 and 299

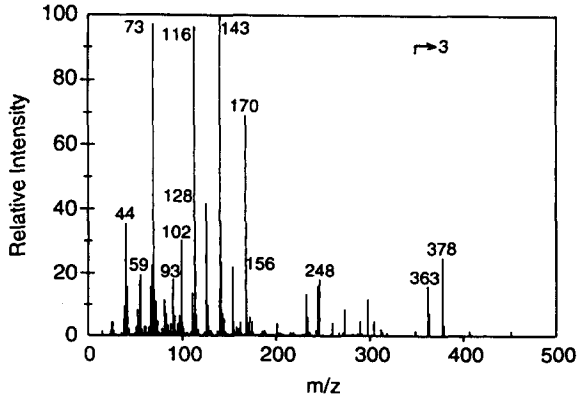

Fig. 2. DIP/EI mass spectrum of tri-TMS derivative of WR-151326.

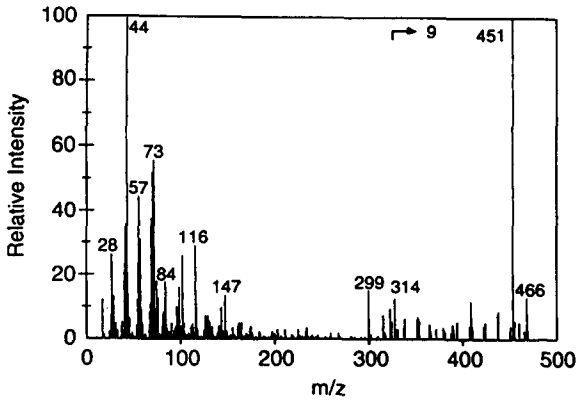

Fig. 3. DIP/EI mass spectrum of di-TMS derivative of WR-25595501.

suggests some remaining traces of phosphoric acid in the sample which were also detected in Fig. 1D as b. On the other hand, the DIP/EI mass spectrum of the underivatized WR-25595501 failed to show a molecular ion at m/z 322 but the spectrum did show other important characteristic ions: m/z at 277 (10%), 147 (100%) and 73 (30%). The degradation of WR-25595501 under mass spectral conditions cannot be ruled out, since it is a polar molecule.

To test the feasibility of simultaneous detection and analysis of mixtures of the aminothiol and its corresponding disulfide, GC calibration graphs for the TMS preparations of the equimolar mixtures of WR-151326 and WR-25595501 were analyzed on a capillary GC column, compound concentrations being plotted against peak areas from the corresponding peak integrations. The lowest concentration of each of the compounds detected was close to 5 pmol. Linear regression was used to analyze the calibration graphs. In both cases, the observed correlation coefficient R^2 was >0.997 and was reproducible, indicating an acceptable relationship for the data ranges of concentration of the compounds examined.

In addition to the GC and MS methodologies, we developed a rapid and sensitive LC–EC methodology for the simultaneous detection and analysis of WR-151326 and WR-25595501 as further confirmation of drug purity and analysis. The LC–EC procedure is similar in principle to that described by McGovern et al. [16] for WR-1065 and by Pendergrass et al. [17] for glutathione. The procedure did not require derivatization of the samples prior to analysis and took less than 15 min to complete.

Table 2
WR-151326 and WR-25595501: redox state, sensitivity and detection limit

Parameter	WR-151326 (WR-151326 [a])	WR-25595501 (WR-25595501 [a])
Redox state [b]	>29 ($>16 \times 10^8$)	$<4 \times 10^{-9}$ ($<4 \times 10^{-9}$)
Slope [c]	162	70
x-Axis intercept [d]	132	23

[a] Synthesized thiol and disulfide compounds.
[b] Ratio of thiol to disulfide.
[c] Detector sensitivity (pA $pmol^{-1}$).
[d] Limit of detection (pmol).

External calibration graphs for WR-151326 were linear over the range 156–2500 pmol injected (slope, 162 nA $pmol^{-1}$; $R^2 > 0.999$). The disulfide, WR-25595501, showed a linear response from 39 to 2500 pmol injected (slope, 70.4 nA $pmol^{-1}$; $R^2 > 0.999$). These data are summarized in Table 2. The active forms of WR-151327, whose specific syntheses are described here, are indicated in Table 2.

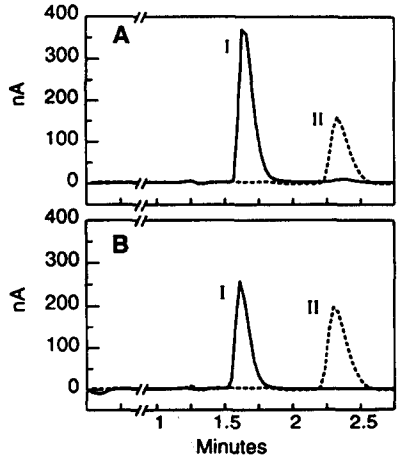

Fig. 4. LC–EC profiles of authentic samples of (A) WR-151326 and WR-25595501 and (B) newly synthesized compounds. I, WR-151326 (solid line); II, WR-25595501 (dashed line).

As can be seen in Fig. 4, the chromatograms for the synthesized compounds (B) are superimposable over the chromatograms for the authentic samples (A). The purity of each synthetic compound was confirmed by estimation of its redox state (the ratio of its thiol to disulfide content). The authentic thiol compound exhibited traces of its symmetrical disulfide, presumably due to autooxidation. However, both the newly synthesized disulfide preparation and the WRAIR (authentic) disulfide preparation were essentially free of LC–EC-detectable contaminating species. (Authentic samples of thiol and disulfide were desiccated and stored at −20°C.) The detection limit (signal-to-noise ratio >3) was 156 pmol for the thiol and 39 pmol for the disulfide.

The procedures for the conversion of WR-151327 into WR-151326 and WR-25595501 described in this paper provide overall high yields and purities of products. The mechanistic pathway for the formation of WR-151326 from WR-151327 is presumably analogous to that described by Risley et al. [9] for the formation of WR-1065 from the hydrolysis of WR-2721. Hydrolysis of the phosphorothioate compound results in the cleavage of the S–P bond to yield thiol and inorganic phosphate:

$$R\text{–}S\text{–}PO_3H_2 + H_2O \rightarrow R\text{–}S\text{–}H + H_3PO_4 \quad (1)$$

The formation of disulfide by iodine-induced oxidation of WR-151326 can best be explained as a two-electron displacement process:

$$R\text{–}S^- + I_2 \rightarrow R\text{–}S\text{–}I + I^- \quad (2)$$

$$R\text{–}S^- + R\text{–}S\text{–}I \rightarrow R\text{–}S\text{–}S\text{–}R + I^- \quad (3)$$

However, metal ion–oxygen-induced oxidation of WR-151326 is best considered as a one-electron process as described by the following sequence of reactions:

$$R\text{–}S^- + M^{+2} \rightarrow R\text{–}S^{\cdot} + M^+ \quad (4)$$

$$R\text{–}S^{\cdot} + O_2 \rightarrow R\text{–}S\text{–}O\text{–}O^{\cdot} \quad (5)$$

$$R\text{–}S\text{–}O\text{–}O^{\cdot} + R\text{–}S\text{–}H \rightarrow R\text{–}S\text{–}O\text{–}O\text{–}H + R\text{–}S^{\cdot} \quad (6)$$

$$R\text{–}S^{\cdot} \rightarrow R\text{–}S\text{–}S\text{–}R \quad (7)$$

The results indicate that both the GC and LC–EC methods described in this paper are specific and sensitive techniques for the simultaneous detection of active metabolites of WR-151327, with the estimate for the lower limis of detection in the pmol range. Although the GC method appears to be more sensitive than LC–EC, both methods are reliable and provide reproducible linear calibration graphs for a relatively wide range of concentrations of the active forms. The linearity of the calibration graphs observed from the GC and LC–EC analyses suggest that these methods may be useful for support of bulk drug studies. However, a more rigorous validation needs to be performed for use of these procedures in toxicological studies demonstrating specificity (i.e. blank plasma versus low-level spiked standards), precision and accuracy including drug stability.

4. Conclusions

The analytical procedures described herein for the active metabolities of WR-151327 will be useful to investigators working in the area of radio- or chemoprotection with in vivo and in vitro studies. Future research is essential in order to continue to evaluate chemo- and radioprotective properties of these aminothiols and symmetrical disulfide class of compounds having structural similarity to the benchmark radioprotector WR-2721 and its active metabolites. The GC, LC–EC and MS procedures described for the analysis of the active metabolites of WR-151327 may be useful in evaluating many compounds of this class.

Acknowledgments

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