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Stability-indicating HPLC assay and solution stability of a new diaziridinyl benzoquinone

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Abstract

RH1, 3-methyl-6-hydroxymethyl-2,5-diaziridinyl-1,4-benzoquinone, is a NQO1 (NAD(P)H quinone oxidoreductase) directed anti-tumor agent. It is designed as a water soluble analog of MeDZQ (3,6-dimethyl-2,5-diaziridinyl-1,4-benzoquinone) and is a drug candidate for clinical evaluation. A HPLC assay has been developed for its analysis. The assay is sensitive (ldl < 0.2 ng), precise (rsd $< 1\%$), linear ($r^2 = 0.9997$), accurate (error $< 0.6\%$), and stability-indicating. Using the developed assay, aqueous stability of RH1 has been evaluated. Both aziridine rings in MeDZQ are known to be easily hydrolyzable in aqueous solutions, however, hydrolysis of the second aziridine ring in RH1 appears inhibited.

Keywords: RH1; 3-methyl-6-hydroxymethyl-2,5-diaziridinyl-1,4-benzoquinone; HPLC assay; Aqueous stability; Decomposition products

1. Introduction

Many cancerous cells, such as lung, colon, liver, and breast, have elevated levels of NAD(P)H quinone oxidoreductase (NQO1). Up to 80 and 35 fold increased expression of NQO1 is observed, respectively, in tissues of non-small and small cell lung cancers relative to the normal [1–3]. NQO1 reduces certain benzoquinones, particularly the diaziridinylbenzoquinones (Fig. 1, DAQ), to their hydroquinones which alkylate and crosslink DNA, resulting in cytotoxicity [4–8]. For this reason, DAQ such as 3,6-bis(carboethoxyamino)-2,5-diaziridinyl-1,4-benzoquinone (Fig. 1, AZQ), 3,6-dimethyl-2,5-diaziridinyl-1,4-benzoquinone (Fig. 1, MeDZQ) and 3-(2-carbamoyloxy-1-methoxyethyl)-6-methyl-2,5-diaziridinyl-1,4-benzoquinone (Fig. 1, CQ, carboquone) have been synthesized as NQO1-directed anti-tumor agents [9–12]. However, they have poor aqueous solubility and are difficult to be formulated. QSAR studies indicate that the anti-tumor activity of DAQ bears a negative correlation to their log *P* [13]. Thus, a water soluble 3-methyl-6-hydroxymethyl-2,5-diaziridinyl-1,4-benzoquinone (RH1), which is suitable for intravenous administration

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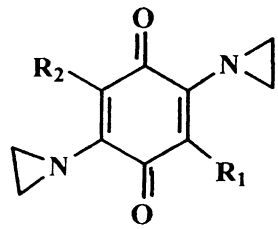

DAQ

	R1	R2
AZQ :	$-NHCO_2CH_2CH_3$	$-NHCO_2CH_2CH_3$
MeZQ :	$-CH_3$	$-CH_3$
CQ :	$-CH(OCH_3)CH_2OCONH_2$	$-CH_3$
RH1 :	$-CH_3$	$-CH_2OH$

Fig. 1. Chemical structure of DAQs.

as a solution, was designed and synthesized as a NQO1-directed anti-tumor agent. Pre-clinical studies suggest that RH1 is highly active and selectively toxic to cells with elevated NQO1 activity [9]. The promise of RH1 as an anti-tumor agent prompted us to develop a HPLC assay that is sensitive and stability-indicating for its analysis.

RH1 belongs to the DAQ class of compounds which is known to be unstable in protic media. The aziridine ring in the DAQ hydrolyses to the ring-opened ethanolamine in neutral or acidic solutions, which is eventually substituted by a hydroxyl group. In basic solutions, it is substituted directly by a hydroxyl group [11,12,14–17]. A stability-indicating HPLC assay for RH1 should separate RH1 from its anticipated hydrolysis products. Several reports appear on the HPLC analysis of DAQ [9,11,14,17,19]. Only one involved the analysis of RH1 [19]. In this report only two small earlier eluting decomposition products were detected. They were presumed by the authors to be the one and two aziridine ring opening products. The basis for their identification was not stated and the stability-indicating capability of the HPLC assay has not been validated.

This paper reports the validation of a specific, sensitive, and stability-indicating HPLC assay for RH1. It also reports the stability of RH1 in aqueous solutions.

2. Experimental

2.1. Reagents and materials

Reagent grade ammonium acetate (NH_4OAc) was obtained from Sigma Chemicals (St. Louis, MO). Reagent grade sodium phosphate monohydrate (KH_2PO_4), and HPLC grade MeOH and

Table 1
Linearity and accuracy data for HPLC validation of RH1 assay[a]

Standard	Peak intensity			mg ml^{-1} of RH1		Error[c]
	RH1	IS	F = RH1/IS	Actual	Found[b]	%
1	1951	2777	0.7025	0.0583	0.0581	−0.28
2	3349	2775	1.2068	0.0970	0.0983	+1.35
3	5358	2769	1.9350	0.1572	0.1563	−0.57
4	6566	2790	2.3537	0.1912	0.1896	−0.78
5	8651	2797	3.0931	0.2473	0.2486	+0.51
					Avg[d]	0.7

[a] See text for HPLC conditions. Linear regression analysis of $F(x)$ vs actual RH1 concentration (y) gave $y = 12.553x–0.0273$, $r^2 = 0.9997$. Standard error of slope and intercept were 0.123 and 0.0202, respectively.

[b] Found RH1 concentration = $(F+0.0273)/12.553$.

[c] Error = (found RH1 concentration − actual RH1 concentration)/actual RH1 concentration × 100.

[d] Avg = $\Sigma|\text{error}|/5$.

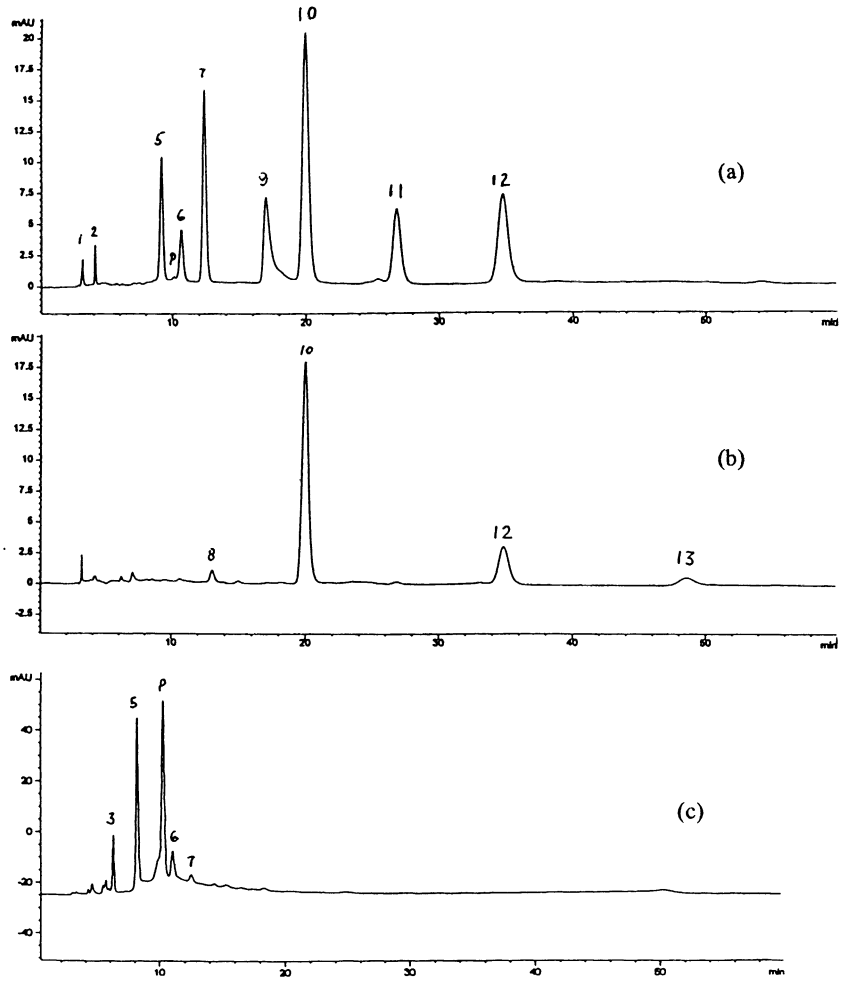

Fig. 2. HPLC chromatograms of a solution of RH1 in (a) 0.1 N HCl, fresh; (b) 0.1 N HCl, one day old; and (c) 0.1 N NaOH, fresh. See text for experimental details.

glacial acetic acid were purchased from Mallinckrodt (Paris, KY). Reagent grade 85% phosphoric acid was purchased from American Scientific Products (McGaw Park, IL). Solutions of 0.1 N HCl and NaOH were prepared from Dilute-it Analytical Concentrate (J.T. Baker, Phillipsburg, NJ). Acid, base and buffer solutions were prepared with H_2O purified through a Millipore Super-Q pure Water System (Waltham, MA).

RH1 samples were received from the National Cancer Institute (Bethesda, MD). ρ-Nitroaniline, the internal standard, was obtained from Eastman Organic Chemicals (Rochester, NY). The internal standard solution (ISS) was prepared by dissolving 13 mg of ρ-nitroaniline per 100 ml H_2O. Unless otherwise mentioned, sample test solutions for assay validation were prepared in the ISS to yield 0.08–0.3 mg ml^{-1} of RH1.

2.2. HPLC

The HPLC assay system consisted of a Waters 600 HPLC pump (Milford, MA), a Thermoseparation (Fremont, CA) AS3000 Autosampler, and

an HP 1050 photodiode array (PAD) detection system (Wilmington, DE). For HPLC assay detection wavelength was set at 330 nm, the absorption maximum of RH1. For monitoring the UV profiles of forced decomposition products and collection and processing of data, the detection system was equipped with a HP 3D ChemStation. Sample and test solutions (20 μl) were loaded on a Phenomenex (Torrance, CA) Inertsil C8, 5 μ, 250 × 4.6 mm i.d. stainless steel column. Chro-

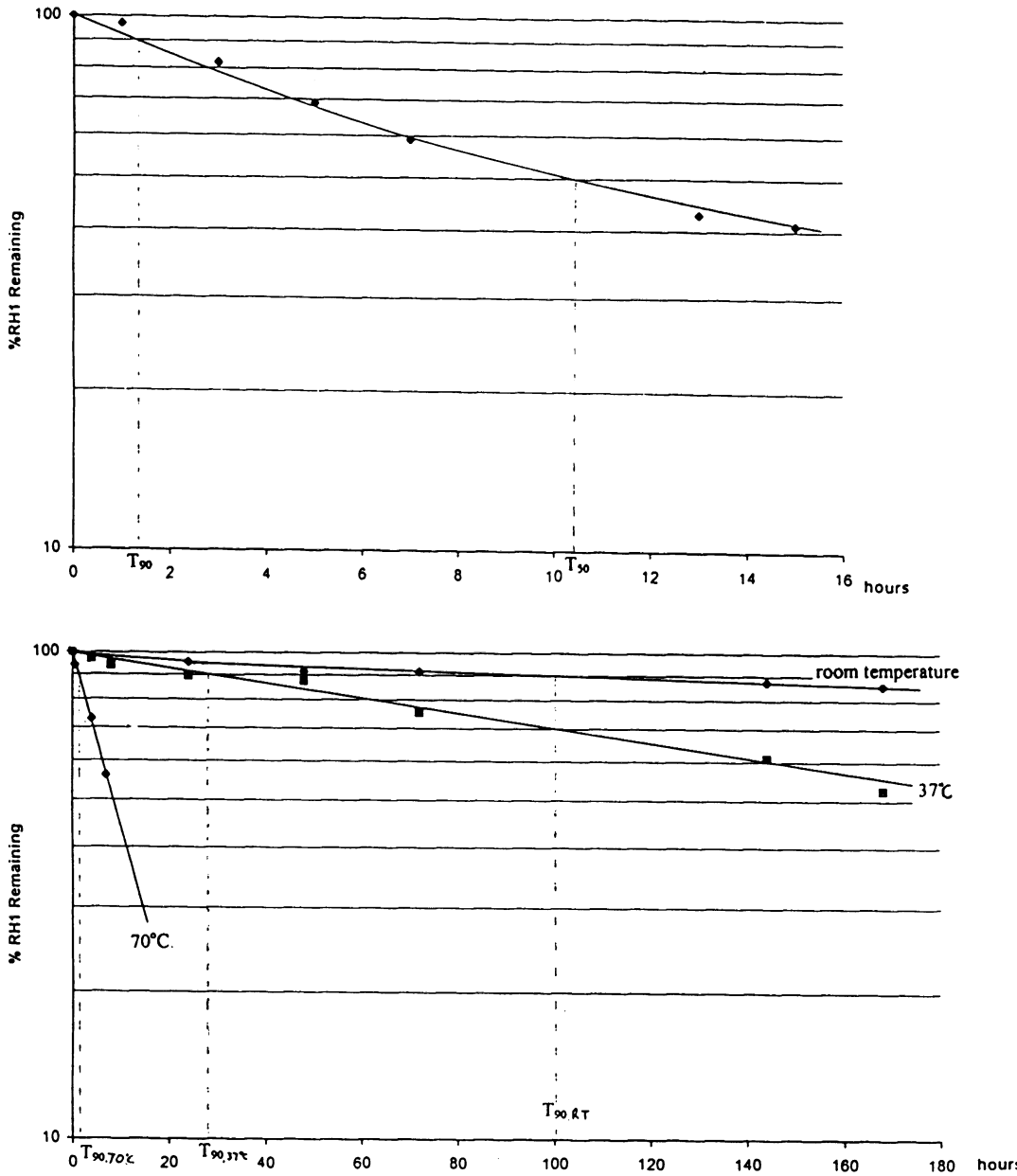

Fig. 3. Stability profile (% remaining vs time plot) of RH1 solution in (a) in 20 mM NaH_2PO_4, pH 4.0, room temperature; (b) in 20 mM NaH_2PO_4, pH 7.0: room temperature, 37 and 70°C; and (c) in 20 mM Na_2HPO_4, pH 10.0, room temperature.

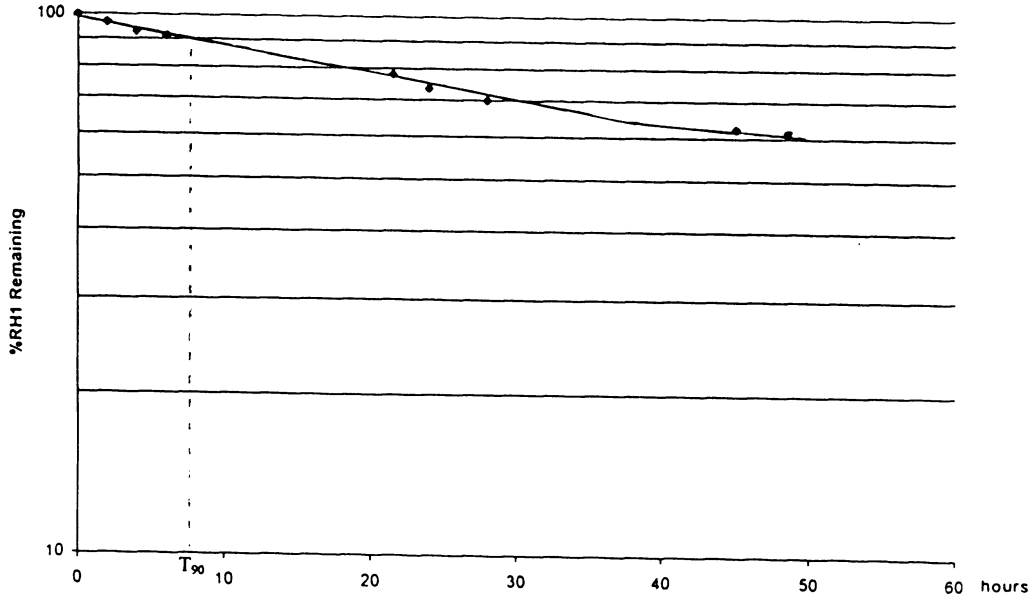

Fig. 3. (*Continued*)

matographic analysis was carried out by isocratic elution with a 30:70 mixture of MeOH and NH_4OAc (pH 4.0; 20 Mm), at 1 ml min^{-1}. LC-MS was performed with a Scientific Systems, Inc. (State College, PA) Model 222/232 HPLC pump system equipped with a Vestec (Houston, TX) LC Thermospray interface and a 201 XL mass spectrometer. Additional LC-MS was performed with a Shimadzu (Columbia, MD) LC-10AD pump with SCL-10AVP System Controller equipped with a Gilson (Middleton, WI) 215 Liquid Handler autosampler and a Perkin-Elmer Sciex (Foster City, CA) API 150EX single quadrupole LC/MS mass spectrometer.

2.3. Solution stability and decomposition products of RH1

Solutions of RH1 were prepared at a concentration of 0.2 mg ml^{-1} in H_2O, 0.1 N HCl, 20 mM NaH_2PO_4 (pH 4.0, 7.0, 10.0), or 0.1 N NaOH. The solutions were left at room temperature on a bench top or, if necessary, heated in a H2025 Temp Blok Module Heater (Scientific Products Division of American Hospital Supply Corp., McGaw Park, IL) at elevated temperatures (37 and 70–75°C) to create forced decomposition products. The 0.1 N NaOH solution was adjusted to pH 4 with H_3PO_4 before HPLC analysis. Peak assignments in the chromatograms were made with their retention times, mass and UV spectral comparisons. At each time point, aliquots of an internal standard solution were individually added to aliquots of the pH 4, 7, and 10 stability solutions just prior to HPLC assay to obtain data for the stability plots (Fig. 3).

3. Results and discussion

3.1. HPLC separation and assay validation

HPLC analysis of RH1 has been reported using a cyanopropyl column [19]. The reported HPLC conditions gave a plate number of $\sim$6400 for the column when calculated from the chromatogram in Reference [19]. Our attempts on the HPLC separation of RH1 suggested that a silica based C8 column would offer better efficiency and peak asymmetry. Under the conditions listed in (Section 2.3), the HPLC assay offered column plate number of 14 000–15 000 and peak asymmetry of

1.1 at 10% height for RH1. A typical RH1 sample contained two tiny impurities (**3**, rT = 0.60; **5**, rT = 0.79; rT is the retention time relative to RH1). Both and many other products could be generated in aqueous solutions upon heating or addition of acid/base. These products were resolved from RH1 (p, rT = 1.00) and the internal standard ρ-nitroaniline (IS, rT = 1.57). Validation

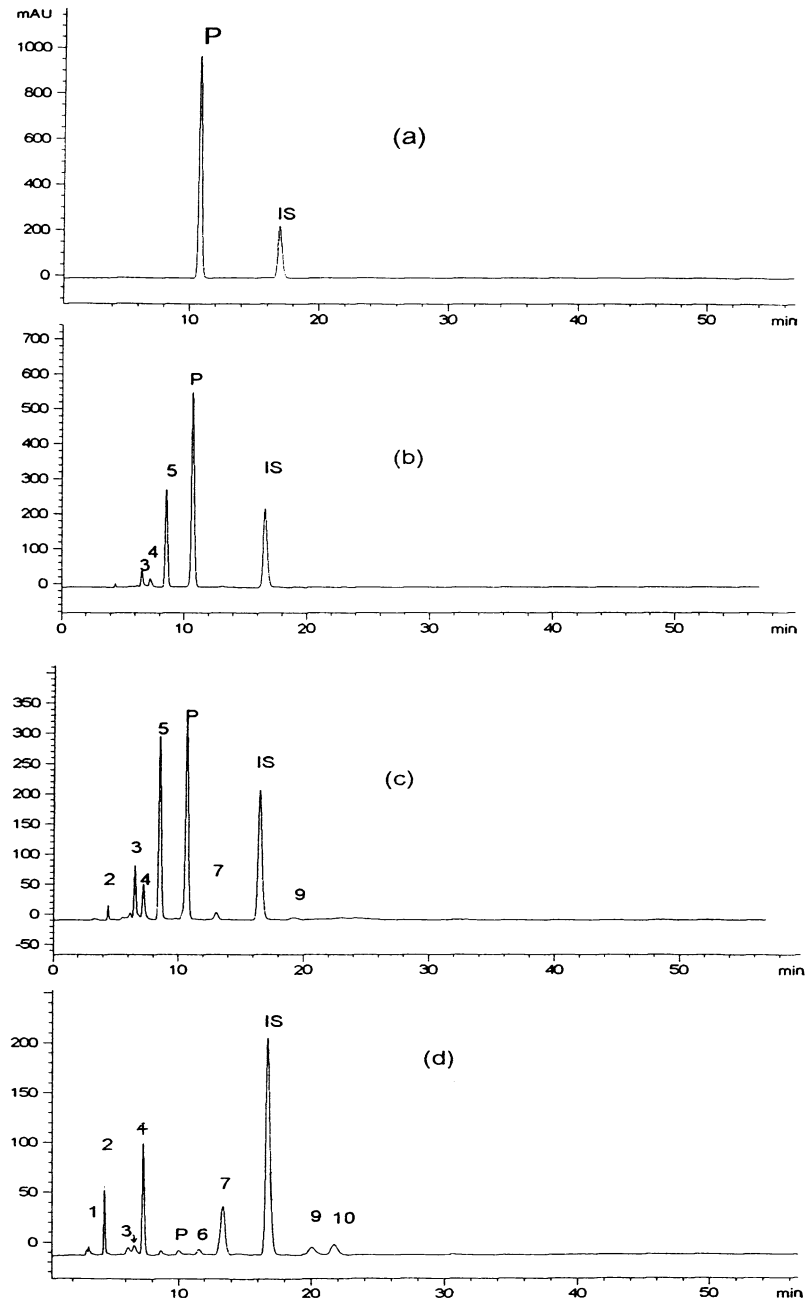

Fig. 4. HPLC chromatogram of a RH1 solution in 20 mM NaH_2PO_4, pH 4.0 at room temperature (a) fresh; (b) 5 h; (c) 16 h; and (d) 7 days. See text for experimental details.

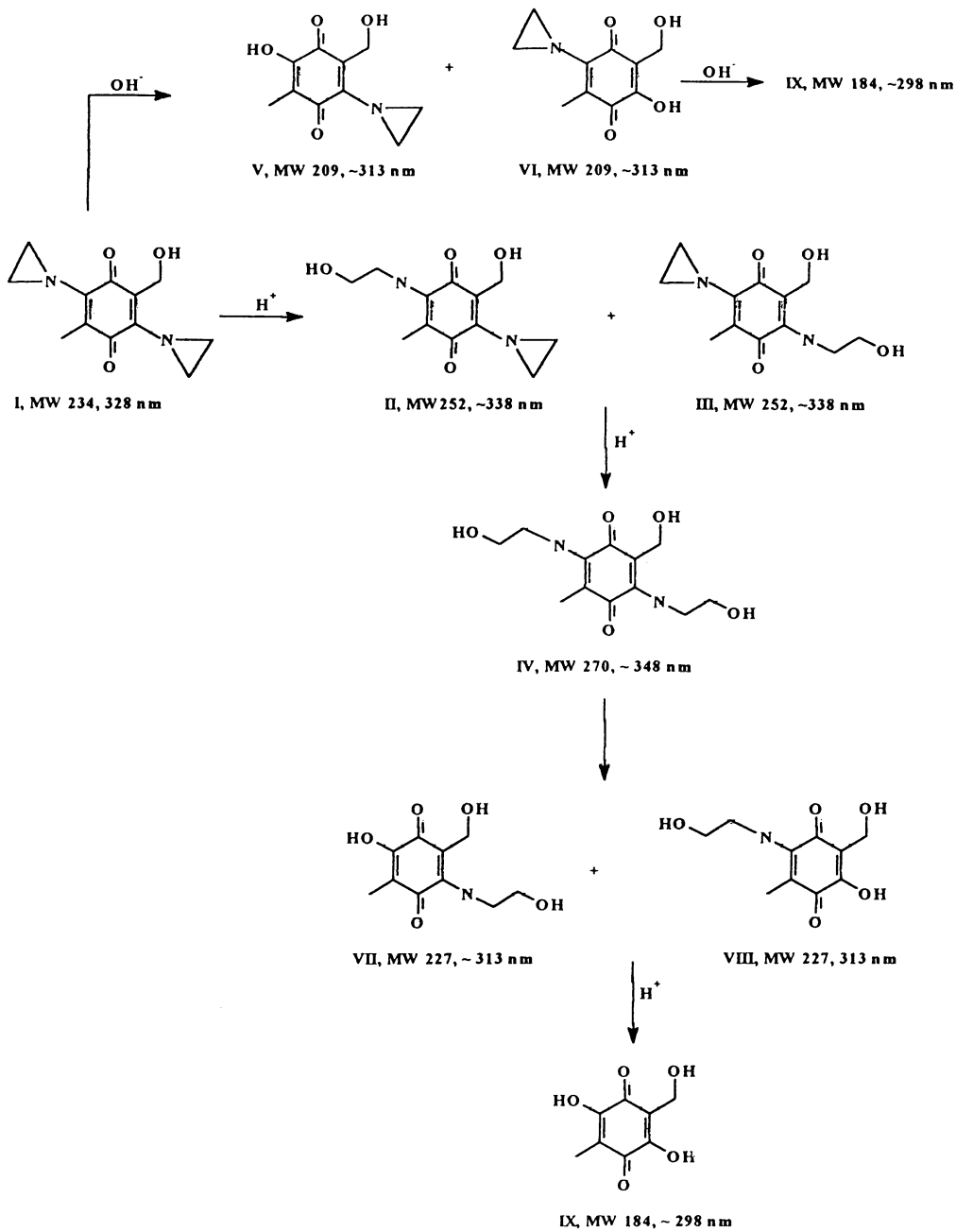

Fig. 5. Anticipated hydrolysis pathway and expected products of RH1.

of the HPLC assay was based on chromatographic data derived from RH1 standard solutions. The repeatability (rsd) obtained from five different standard solutions of similar concentration (140–160 μg ml^{-1} of RH1 in the ISS solution) was 0.8% within day and 0.8% between days ($n = 3$). Based on data from five standard solutions (Table 1), the assay was linear ($r^2 = 0.9997$,

slope = 12.553 with standard error of 0.123, and intercept = −0.0273 with standard error of 0.0202) and accurate (error = 0.7%). Based on a 3:1 signal-to-noise ratio, the lower detection limit (ldl) for RH1 was 1 ng. Accuracy of the assay was further demonstrated by the analysis of two lots of RH1 samples. The purity were established as 97.7% for lot A and 97.4% for lot B, by a material balance consideration of elemental, HPLC impurity, moisture and organic volatile data. The HPLC assay for lot B, using the 97.7% pure lot A as the working standard, is 97.4% ($s = 0.9$, $n = 4$). The assay result is identical to that derived from mass balance consideration.

3.2. Stability of RH1 in aqueous solution

RH1 is extremely unstable in strongly acidic (pH 1) and basic (pH 13) solutions. In 0.1 N HCl, it instantaneously decomposed to at least seven

Table 2
Relative retention (rT) and spectral data, and identities of decomposition products of RH1

Peak	rT	MS data	UV (λ_{max}) nm	Identity (Fig. 5)
1	0.31	210 (M+H)	338	VI
2	0.45	210 (M+H)	322	V
3	0.60	253 (M−H)	347	III
4	0.67	241 (M+H)	350	Unknown
5	0.79	253 (M+H)	340	II
p	1.00	235 (M+H)	328	I, RH1
6	1.07	–	342	Unknown
7	1.22	389 (M+H)	352	Unknown
8	1.31	373 (M+H)	282, 357	Unknown
9	1.88	401 (M+H)	328	Unknown
10	2.06	401 (M+H)	343	Unknown
11	2.59	[a]	339	Unknown
12	3.62	437 (M+H)	285, 362	Unknown
13	4.49	567 (M+H)	347	Unknown

[a] Too short-lived in solution for LC-MS analysis.

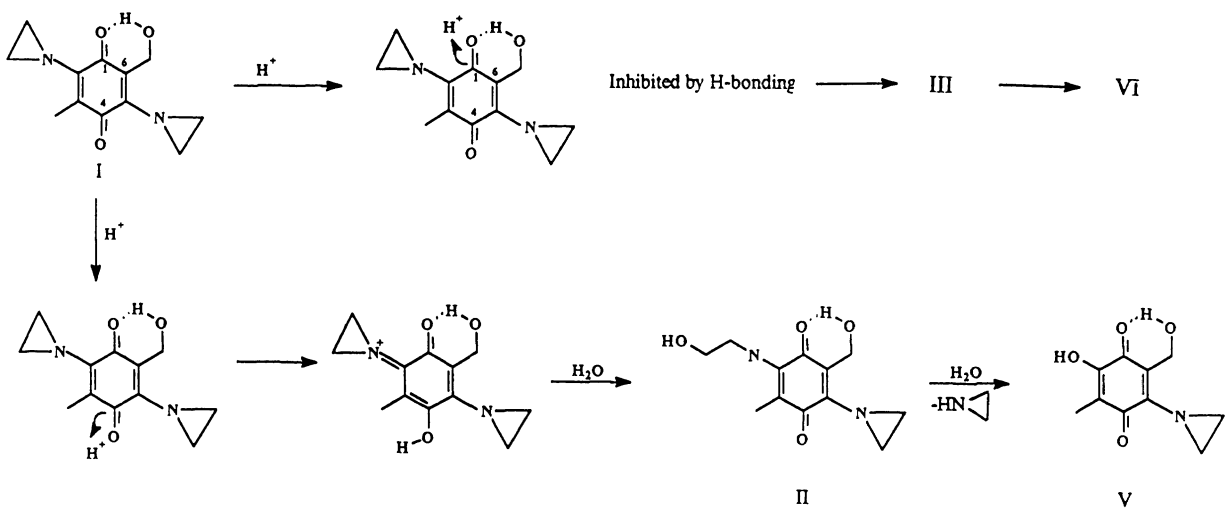

Fig. 6. Proposed hydrolysis inhibition of RH1 due to H-bonding.

products which were mostly later eluting than RH1 (Fig. 2a). Several of the products (**5**–**7**, **9**) were further decomposed to late eluting products after the solution was let stand at room temperature for a day (Fig. 2b). In 0.1 N NaOH, $>50\%$ decomposition was observed instantaneously (Fig. 2c). In place of RH1 were products **3**, **5**, and **6**. In more mildly acidic (pH 4) solutions, RH1 decomposed slowly at room temperature with a T_{90} and T_{50} (time for10 and 50% decomposition) of 1.3 and 10.4 h, respectively (Fig. 3a). Product **5** was the initial major decomposition (Fig. 4(a–b)). As the solution aged at room temperature, **3** and **4** began to be significant (Fig. 4c). After a week, **2**, **4**, **7**, **9** and **10** became the major with **6**, **3** and **1** as minor products (Fig. 4d). Stability of RH1 in H_2O was similar to that in pH 7 buffer with T_{90} of >72 h (Fig. 3b). The stability was very sensitive to temperature, however. The T_{90} was reduced substantially to about 26 and 1 h, respectively, when the neutral solutions were heated at 37 and 70°C. Heating the neutral solution of RH1 resulted in small amounts of **3**, **4** and **5**. In pH 10 buffer, RH1 was more stable than in pH 4 buffer with a room temperature T_{90} of 7 h (Fig. 3c). The products were similar to those in the NaOH solutions, albeit in much smaller amounts. Though the chromatograms presented were detected at 330 nm, detection at 254 nm revealed no additional products.

3.3. Hydrolytic decomposition of RH1

In neutral and acidic aqueous solutions, both aziridine rings of DAQ are known to hydrolyze sequentially via ring opening to the ethanolamines which are subsequently substituted with hydroxyls. While in basic solutions, the aziridine rings are sequentially substituted by hydroxyls [11,12,17,18]. Under reversed-phased HPLC conditions analogous to the ones described in this paper, these hydrolysis products were all eluted ahead of the parent DAQ [12,17]. UV chromophores of DAQ would undergo a red shift of ~ 10 nm for each ring opening and a blue shift of 10–20 nm for each hydroxyl substitution of the aziridine moiety [15,16,18]. RH1 has a molecular weight (MW) of 234 and an absorption maximum (λ_{max}) of 328 nm in the HPLC mobile phase. Thus, eight hydrolysis products (II–IX), depicted in Fig. 5 along with their MW and anticipated λ_{max}, would be expected from RH1. Examination of chromatograms from room temperature and elevated temperature decomposed RH1 solutions shown in Figs. 2 and 4 reveals 13 significant products (**1**–**13**), though not every product is present in all solutions. The relative HPLC retention (rT), MW and λ_{max} of these products are listed in Table 2. MW and λ_{max} data were obtained with LC-MS and LC-PAD. Note that none of the major products has MW of 270, 227 and 184 for IV, VII, VIII, and IX. This suggests that hydrolysis of a second aziridine ring is not significant for RH1. Both aziridine rings in 3,6-dimethyl-2,5-diaziridinyl-1,4-benzoquinone (MeDZQ) have been reported to hydrolyse readily [11,17]. Therefore, hydroxylation of the 6-methyl in RH1 apparently inhibits the hydrolysis of a second aziridine ring and prevents formation of products IV, VII, VIII, or IX. Skibo and Xing [10] reported that acid hydrolysis of the aziridine ring in aziridinylbenzoquinones was initiated by *O*-protonation via an imino intermediate (Fig. 6). Possible H-bonding between the 6-hydroxymethyl and the 1-carbonyl in RH1 may have inhibited the protonation of the 1-carbonyl and slowed down the hydrolysis of the 5-aziridine as shown in Fig. 6.

Of the 13 significant decomposition products of RH1, only four earlier eluting ones (**1**–**3**, and **5**) matches the expected hydrolytic products (Fig. 5). Products **3** and **5** have identical MW of 252 (from LC-MS) and λ_{max} of 347 and 340 nm, respectively. They are consistent with ring opening products II and III, though the λ_{max} of **3** (347 nm) appears longer than expected. In general, **5** was the initial and predominant product. According to Fig. 5, **5** is II and **3** is the inhibited product III. As the decomposition advanced, product **2** and at times **1** (both have identical MW of 209; λ_{max} for **2** and **1** are 322 and 338 nm, respectively) began to appear (Fig. 3c Fig. 4d). Their rT, and MW are consistent with V and VI. The dominance of **2** over **1** suggests that **1** is the inhibited VI. The λ_{max} of **2** (322 nm) is consistent with V. The λ_{max} of **1** (338 nm) is longer than expected of VI, however. The unexpected longer λ_{max} for III and VI could result

from H-bonding contribution. Identities of the remaining products **4** and **6–13** are not directly apparent from their MW and UV data and are not presented in this paper. Their MW (Table 2) do not match with any of the expected hydrolysis products presented in Fig. 5.

4. Conclusion

A rapid and sensitive assay of RH1, a water soluble analog of MeDZQ, has been developed and validated. The assay is stability-indicating. It resolves RH1 from potential decomposition products. Aqueous stability of RH1 has been described. At room temperature, RH1 is instantaneously decomposed in acidic (pH 1) and basic (pH 13) solutions. It is more stable in milder pH (4 and 10) solutions, with T_{90} (10% decomposition) of about 2 and 7 h, respectively. In water or pH 7 buffer, it is fairly stable with T_{90} of >72 h. As RH1 has substantial aqueous solubility (>5 mg ml^{-1}) and stability at neutral pH, it will likely be formulated as a freeze dried powder for reconstitution with normal saline or 5% dextrose solution. Though numerous hydrolytic products have been detected for RH1, none corresponds to the opening or hydroxyl substitution of both aziridine rings.

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