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# Preformulation studies to aid in the development of an injectable formulation of PD 144872, a radiosensitizing anticancer agent

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## Summary

PD 144872, the *R*-enantiomer of $\alpha$-[[(2-bromoethyl)amino]methyl]-2-nitro-1H-imidazole-1-ethanol hydrobromide, is a dual-action hypoxic cell radiosensitizer which is currently being evaluated at the preclinical level. This study was performed to aid in the development of a parenteral dosage form of PD 144872 for use in toxicologic and clinical testing. PD 144872 shows good solid-state stability under accelerated storage conditions; however, it shows unacceptable stability (for the formulation of a ready-made solution dosage form) in aqueous solution at 25 and 30°C. The pH-$k_{obs}$ profile is well described by a pathway involving water-catalyzed or spontaneous degradation of the neutral species which implies that PD 144872 is most stable under acidic conditions. Unlike melphalan, co-solvent systems do not stabilize the compound. The postulated degradation mechanism is intramolecular, nucleophilic attack by the amino group at the $\beta$-carbon to form the corresponding aziridine derivative which can be opened, in a subsequent step, by water or other suitable nucleophiles. The absence of racemization was confirmed by monitoring the chirality of the aziridine product formed upon the degradation of to PD 144872. The apparent solubility of PD 144872, in the pH range of 2–4, is independent of pH and suggests that the limiting solubility of the cationic form is about 50 mg/ml at 25°C and about 33 mg/ml at 4°C. Based on these studies, formulation efforts will focus initially on the development of a lyophilized vial where manufacture and reconstitution will be conducted at pH $\leq$ 3 and where the maximum reconstitution concentration is kept below 50 or 33 mg/ml when stored at 25 or 4°C, respectively. This should provide optimal stability and solubility conditions.

## Introduction

Many solid tumors contain hypoxic cells which are more resistant than oxygenated cells to the lethal effects of radiation and which have been postulated to play a role in the recurrence of the tumor following radiotherapy. The sensitivity of hypoxic cells to radiation can be enhanced by the use of chemical radiosensitizers (Shenoy and Singh, 1992). For example, 2-nitroimidazoles act primarily by a free-radical mechanism (Adams et al., 1975) and have been classified as oxygen-mimetic radiosensitizers (Suto, 1991). Although several 2-nitroimidazoles show radiosensitizing activity in hypoxic cells in vitro, relatively few show activity at nontoxic doses in vivo (Binger and Workman, 1991). An exception is the mixed-

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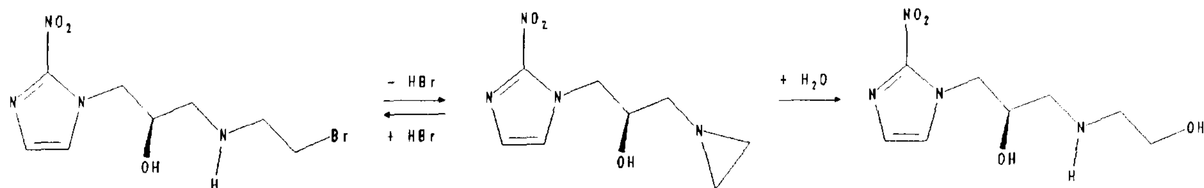


Scheme 1. Postulated mechanism for the degradation of PD 144872. (Although water is depicted, other suitable nucleophiles can also open the aziridine ring.)

function hypoxic cell radiosensitizer, PD 144872, the *R*-enantiomer of α-[[(2-bromoethyl)-amino]-methyl]-2-nitro-1H-imidazole-1-ethanol hydrobromide (see Scheme 1). It contains both a sensitizing nitro group and a 2-bromoethylamino group. This latter group is a promoiety of the alkylating aziridine group and makes PD 144872 a prodrug of the more toxic but slightly more active RSU 1069, α-(1-aziridinylethyl)-2-nitro-1H-imidazole-1-ethanol (Walton and Workman, 1988; Binger and Workman, 1991; Wong et al., 1991).

The bromo analogue of PD 144872 was chosen for development, instead of the chloro analogue, because of its greater radiosensitization efficiency which correlated to its more rapid conversion to the active aziridine product (Jenkins, 1990). Additionally, the *R*-enantiomer of the bromo analogue, was selected instead of the *S*-enantiomer or the racemate, because of its lower emetic potential; emesis can be a potentially dose-limiting toxicity. The current study was undertaken to aid in the development of a suitable parenteral dosage form of PD 144872 for use in toxicologic and clinical testing.

## Materials and Methods

### *Materials*

PD 144872, the corresponding aziridine (as the racemate and pure enantiomers), and the aquated analogue were synthesized by the Chemistry Department at Parke-Davis Pharmaceutical Research (Ann Arbor, MI). All other chemicals were of reagent or analytical grade, and the water was distilled and deionized.

### *Analytical methods*

The pH measurements were performed at the temperatures of the studies with an Accumet pH meter 915, using a two-point calibration, and a Ross combination glass electrode. The achiral HPLC analyses were performed on an HP 1090 liquid chromatograph equipped with a diode-array detector operating at a fixed wavelength of 325 nm, a $\lambda_{max}$ for PD 144872 dissolved in mobile phase. The column was a Supelcosil LC-8 (15 cm × 4.6 mm) 5 μm column. The mobile phase was composed of a 25:75 mixture of methanol:aqueous phase. The aqueous phase contained 1% (v/v) of triethylamine, 1 mg/ml of the sodium salt of 1-decanesulfonic acid, and 50 mM ammonium phosphate; the pH of this phase was adjusted to 3.0 with 85% phosphoric acid to ensure that no PD 144872 degradation occurred in the mobile phase during analysis. The injection volume was 20 μl; the eluent flow rate was 1.5 ml/min; and the run time was 10 min. (Since the time of this work, it has been found that acetonitrile is better than methanol in the resolution of the aziridine and aquated products shown in Scheme 1.)

The enantioselective HPLC assay differs from the achiral method as follows: the column was a Chiralpak AS (Amylose tris((*S*)-α-methylbenzyl carbamate)) (25 cm × 4.6 mm) 10 μm column; the mobile phase was composed of 200 parts of hexane, 100 parts of ethanol, and 0.2 parts of triethylamine (by vol.); the detection wavelength was

315 nm (a $\lambda_{max}$ for the aziridine under these conditions); and the eluent flow rate was 1 ml/min.

*Solubility studies*

The aqueous solubility of PD 144872 was determined as a function of pH at approx. 4°C (the temperature varied from 3 to 7°C over the 24 h period) and 25°C. The pH was maintained with either HCl or 100 mM formate buffers. Excess solid PD 144872 ($\sim$60 mg/ml) was placed in screw-capped vials to which the media of interest were added. For the 25°C studies, the vials were rotated at 75 rpm on a rotating-bottle apparatus which was set in a water bath. For the 4°C studies, the vials were shaken in an electronic shaker which was set in a refrigerator. At predetermined times, 1-ml samples were removed from the vials and filtered through 13-mm, 0.45-$\mu$m Acrodisc LC13 PVDF (polyvinylidene difluoride) syringe filters. At least 0.5 ml of the mixture was used to wash the filter, before the filtrate was collected for quantitation of PD 144872 and determination of pH. (The measured PD 144872 concentration was independent of the volume passed through the filter, consistent with the absence of filter adsorption.) The 25°C vials were sampled over a 2–6 h period to minimize degradation, whereas the 4°C vials were sampled at 24 h. Prior to HPLC analysis, the samples were diluted by a factor of 100 with 0.01 N HCl. In addition to providing the dilution effect, the 0.01 N HCl was used to quench the degradation reaction.

*Solid-state stability studies*

Solid-state stability was determined by placing about a 200 mg dispersed layer of PD 144872 in open, glass, Petri dishes. Then these dishes were stored in temperature-controlled ovens set at 30, 45, and 60°C and also at 37°C with 75% relative humidity. Over a 6 month interval, approx. 25-mg aliquots of each sample were removed and dissolved in 100 ml of 0.01 M HCl. The resulting solutions were assayed, by comparison to a fresh standard, by HPLC.

*Solution stability studies*

The kinetics of degradation of PD 144872 (0.25 mg/ml) were determined as a function of pH at 4, 25, and 30°C and at an ionic strength of 0.15 M (to approximate isotonic conditions). Additionally, the kinetics at 25°C were studied as a function of PD 144872 concentration and in the presence of ethanol/propylene glycol/water mixtures, whereas those at 30°C were studied as a function of buffer concentration. The buffer species used were formate, acetate, and phosphate; sodium hydroxide was used to attain pH values greater than 11. The kinetic experiments were started by adding 0.10 ml of a stock solution of PD 144872 in 50 mM HCl to 10-ml volumetric flasks containing the reaction mixtures, which were pre-equilibrated at temperature of interest in a circulating water bath. The pH values of these mixtures were measured at the end of each kinetic run.

At predetermined times, samples were withdrawn, quenched with either 1.0 or 0.1 M HCl so that the pH of the resulting solution was less than 2, and assayed for PD 144872 and degradants by achiral HPLC. For the kinetic runs conducted in formate buffer, the samples were refrigerated after acidification until all samples for a given kinetic run were ready to be analyzed. The observed rate constants, $k_{obs}$, were obtained by following the disappearance of the peak area of PD 144872 for typically 3–4 half-lives. (For the kinetic studies performed at 4°C, the reaction was followed for $< 1$ half-life.) In acetate buffers, the reported $k_{obs}$ represent the buffer-independent values. These were obtained by extrapolating the buffer-dependent rate constants to a buffer concentration of zero. The rate and ionization constants comprising $k_{obs}$ were generated by nonlinear least-squares regression of Eqn 1 and the experimental data using PCNONLIN (SCI, Lexington, KY) and the Nelder-Mead simplex algorithm.

To determine if PD 144872 is susceptible to racemization, the degradation of PD 144872 (25 mg/ml) was monitored in 100 mM phosphate buffer at 25°C. The kinetic experiments were started by dissolving 250 mg of PD 144872 in 10-ml volumetric flasks containing the buffer so-

lutions, which were pre-equilibrated at 25°C in a circulating water bath. The pH values of these mixtures were measured after the 24 h sample was pulled. At predetermined times, 100-$\mu$l samples were withdrawn, diluted to 10 ml with ethanol, and assayed for the presence of the *R*- and *S*-enantiomers of the aziridine product by enantioselective HPLC.

Lastly, the apparent activation parameters for the degradation reaction were determined by following the loss of PD 144872 in 10, 25, and 50 mM NaOH at 10, 20, 30, and 40°C.

## Results and Discussion

### *Solubility*

Using a polarizing optical microscope, PD 144872 was found to exist as large plates that were highly birefringent, indicating a high degree of crystallinity. The apparent solubility of PD 144872, over a pH range of 2–4, as a function of the equilibration time at 4 and 25°C is shown in Table 1. The results suggest that the limiting solubility of the cationic form of PD 144872 is approx. 33 mg/ml at 4°C and 50 mg/ml at 25°C. At 25°C, the solubilities in the formate buffers were determined within 2 h to minimize any adverse effects due to the degradation of PD 144872 and the formation of the degradation products; however, the solubility determinations in 0.01 N HCl over a 6 h interval suggest that 2 h is sufficient time to attain equilibrium solubility. The solubility of PD 144872 was not determined at pH greater than 4 because of its facile degradation.

TABLE 1

*Apparent solubility of PD 144872 as a function of pH, temperature, and equilibration time*

| Media | Temperature (°C) | pH | Equilibration time (h) | PD 144872 (mg/ml) |
|---|---|---|---|---|
| HCl | 25 | 2.10 | 2 | 52 |
| | | | 4 | 51 |
| | | | 6 | 49 |
| Formate buffer [a] | 25 | 3.10 | 0.5 | 54 |
| | | | 1 | 53 |
| | | | 2 | 52 |
| | 25 | 3.45 | 0.5 | 54 |
| | | | 1 | 53 |
| | | | 2 | 50 |
| | 25 | 3.97 | 0.5 | 53 |
| | | | 1 | 51 |
| | | | 2 | 49 |
| | 4 | 2.93 | 24 | 33 |
| | 4 | 3.44 | 24 | 33 |
| | 4 | 3.89 | 24 | 31 |

[a] These samples typically had less than 1% degradation by peak area normalization.

### *Solid-state stability*

The solid-state stability of PD 144872 was monitored over a 6 month period. The assay results relative to a fresh PD 144872 standard and the absence of degradant peaks on the chromatograms supported the lack of PD 144872 degradation in the samples stored at 30, 45, and 60°C. The sample stored at 37°C and 75% RH was followed for 2 months. The 2-month results suggested that 98.8% of PD 144872 remained in the sample; however, this percentage change was predominantly due to moisture uptake and not to drug loss via degradation as demonstrated by a minor degradant peak ($\sim$ 0.1% by peak area normalization).

### *Solution stability*

The kinetics of degradation of PD 144872 were studied as a function of pH at 4, 25, and 30°C and at an ionic strength of 0.15 M (with NaCl) with an achiral HPLC assay. Fig. 1 shows the dependence of the degradation rate constant, $k_{obs}$, on pH; PD 144872 was found to be most stable in very acidic pH and least stable at pH values greater than about 9 where the degradation rate was independent of pH. The disappearance of PD 144872 under all conditions was well described by first-order kinetics as seen in Fig. 2.

Over the pH range investigated, a mechanistically consistent expression, relating the buffer-independent, first-order rate constant to the hydrogen-ion activity, can be derived:

$$k_{obs} = k_0\left(\frac{K_a}{a_H + K_a}\right) \tag{1}$$

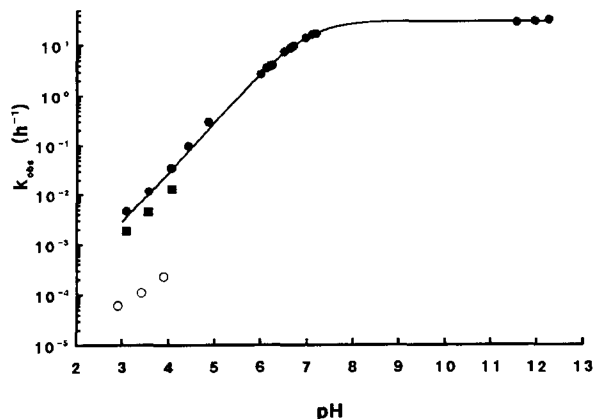


Fig. 1. The pH dependence of the rate constants for the degradation of PD 144872 at 30°C (●), 25°C (■), and 4°C (○) at $\mu = 0.15$ M. The theoretical profile was generated with Eqn 1 and the constants shown in the text.

where $a_H$ is the hydrogen-ion activity, $K_a$ is the ionization constant of the amino group, and $k_O$ is the rate constant for water-catalyzed or spontaneous degradation of the neutral species. (This rate constant is kinetically equivalent to the one describing hydroxide-ion catalyzed degradation of the cationic species.)

The theoretical profile generated at 30°C, shown in Fig. 1, was constructed with a $k_O$ of 30.0 ($\pm$0.4) $hr^{-1}$ and a $K_a$ of $9.53 \times 10^{-8}$ or, alternatively, a $pK_a$ of 7.02. This kinetically generated $pK_a$ value is consistent with the previously

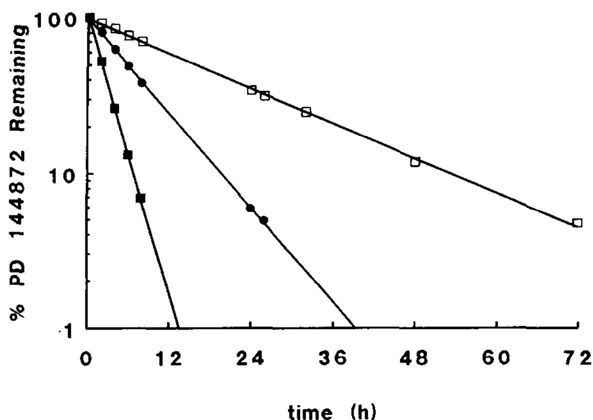


Fig. 2. Representative first-order plots of log % remaining of PD 144872 vs time at $\mu = 0.15$ M and 30°C in 100 mM acetate buffer of pH 4.04 (□), 4.42 (●), and 4.86 (■).

TABLE 2

*pH dependence of several kinetic parameters for the degradation of PD 144872 in formate buffers at 25°C*

| pH | Temperature (°C) | $k_{obs}$ ($h^{-1}$) [a] | $t_{90}$ [b] | % degraded after 8 h |
|---|---|---|---|---|
| 2.90 | 4 | $5.97(\pm 2.21)\times 10^{-5}$ | 73 days | 0.05 |
| 3.41 | 4 | $1.09(\pm 0.21)\times 10^{-4}$ | 40 days | 0.09 |
| 3.89 | 4 | $2.25(\pm 0.18)\times 10^{-4}$ | 19 days | 0.18 |
| 3.09 | 25 | $1.86(\pm 0.01)\times 10^{-3}$ | 57 h | 1.5 |
| 3.55 | 25 | $4.66(\pm 0.09)\times 10^{-3}$ | 23 h | 3.7 |
| 4.03 | 25 | $1.33(\pm 0.02)\times 10^{-2}$ | 8 h | 10.1 |

[a] The values in parentheses are the standard errors. [b] The time required for 10% degradation of PD 144872.

predicted value of 7.2 (Jenkins et al., 1990). Attempts to confirm this value by other techniques were thwarted by the facile degradation of PD 144872 at pH greater than 4.

Table 2 lists the observed rate constants which were determined over a pH range of 3–4 and at 4 and 25°C. Also listed are the $t_{90}$ values (i.e., the time required for 10% degradation of PD 144872) and the percent degraded after 8 h. 8 h was chosen because it is an in-house specification for the maximum time period over which an unpreserved, reconstituted product can be used. The deviation of the slope of the log $k_{obs}$ vs pH data, generated at 4°C, from the theoretical slope of 1 is most likely due to the large standard errors associated with the rate constants since the reactions were followed for 8, 12, and 23% of degradation at pH 2.90, 3.41, and 3.89, respectively, due to the relatively slow rates.

Altering the concentrations of the formate and phosphate buffers had no appreciable effect on the degradation rate, consistent with the absence of buffer catalysis. In contrast, acetate buffers catalyzed the degradation rate and the reaction appears to be subject to only general-base catalysis. Additionally, the formation of a new product was consistent with the fact that acetate ions can compete with water as a nucleophile in the opening of the aziridine ring (Earley et al., 1958). In generating the pH-rate profile, the use of carbonate buffers was avoided because PD 144872 is known to form the oxazolidin-2-one analogue

(Jenkins et al., 1990) which competes with aziridine formation; this reaction has been shown to occur with other simple 2-haloethylamines (Rauen et al., 1965).

The kinetics of degradation of PD 144872 were evaluated in co-solvent systems containing 10% ethanol USP, varying amounts of propylene glycol (PG) USP (ranging from 0 to 30% v/v), and water. Increasing the percentage of PG from 0 to 10% had little to no effect on the stability of PD 144872, whereas increasing the percentage of PG from 10 to 30% resulted in about a 1.3-fold increase in the degradation rate. The increased rate may result from changes in the apparent pH of the media and/or a PG-induced increase in the apparent $K_a$ of PD 144872 as observed with the weak base, triethanolamine (Rubino, 1987). The effect of an increase in the $K_a$ or a change in the hydrogen-ion activity on $k_{obs}$ can be deduced from Eqn 1.

Unlike PD 144872, the use of a co-solvent system dramatically improved the stability of melphalan (Flora et al., 1979) which degrades by a similar mechanism. For melphalan where the nucleophilic nitrogen is tertiary, degradation results in the formation of a charged aziridinium intermediate as opposed to the uncharged aziridine intermediate formed upon the degradation of PD 144872 (see Scheme 1). Relative to water, a co-solvent system results in a decrease in the polarity of the reaction medium, and, hence, a decrease in the ability of the medium to accommodate charged species. Therefore, a co-solvent system should result in greater stabilization of melphalan since its transition state must reflect some of the charge formation that occurs during degradation. In addition to the polarity effects, the enhanced stability of melphalan was due, in part, to the high chloride-ion content of the co-solvent system (Flora et al., 1979).

The effect of the PD 144872 concentration, ranging from 10 to 40 mg/ml, on the kinetics of degradation of PD 144872 was studied in 100 mM formate buffer at pH 3 and 25°C. The results were complicated by the pH decreases which accompany PD 144872 degradation and which were consistent with the loss of HBr upon aziridine formation. However, taking the pH changes into account, the results, as expected for an intramolecular reaction, suggested that altering the initial PD 144872 concentration had no significant effect on the degradation rate. Similarly, mannitol, which may be needed as a bulking agent for a lyophilized formulation, had no effect on the degradation rate.

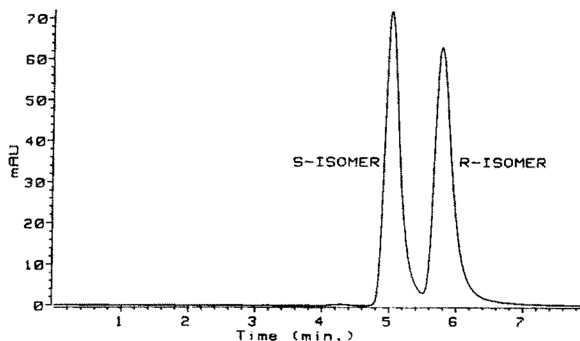


Fig. 3. Enantioselective HPLC assay of a 1.2 mg/10 ml solution of the racemic aziridine product in ehtanol. The assignment of the *S*- and *R*-isomers was made by injection of authentic samples of each enantiomer.

The importance, in the development of chiral drug products, of assessing the ability of enantiomers to interconvert has been addressed previously (De Camp, 1989; Holmes et al., 1990; Wozniak et al., 1991). The susceptibility of PD 144872 to racemization was evaluated by monitoring the chirality of the aziridine formed upon degradation of PD 144872 (25 mg/ml) in 100 mM phosphate buffers at 25°C. (The separation of the *R*- and *S*-isomers by the chiral HPLC method is shown in Fig. 3 which is the chromatogram for the racemic aziridine.) Over 24 h and a pH range of 4.29–4.99, only the *R*-isomer, and not the *S*-isomer, of the aziridine product was observed. Since a fair amount of PD 144872 was still in these solutions, the rate of degradation of PD 144872 was increased by alkalinization with NaOH. Once again, the more completely degraded samples showed none of the *S*-isomer of the aziridine product. These results show that racemization of PD 144872 in aqueous solutions does not occur.

*Mechanism of degradation*

The apparent activation parameters for the water-catalyzed or spontaneous degradation of

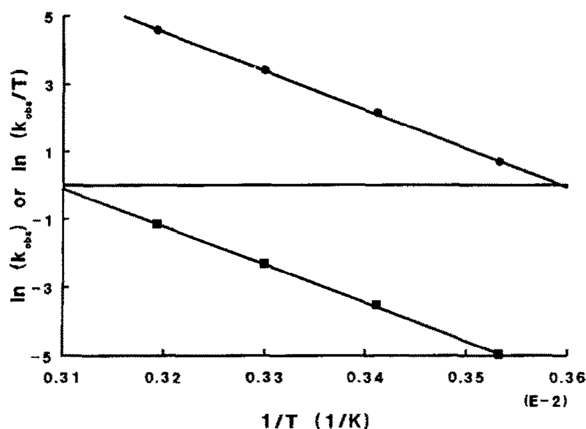


Fig. 4. The Arrhenius (●) and Eyring (■) plots for the degradation of PD 144872. (The rate constants are $h^{-1}$.)

the neutral species of PD 144872 were determined from the effect of temperature on the $k_{obs}$ values in the plateau region of the pH-rate profile (pH $> 11$). These $k_{obs}$ values can be used directly because, as seen in Eqn 1, the smaller $a_H$ becomes relative to $K_a$, the more closely $k_{obs}$ approximates $k_o$. Fig. 4 shows the corresponding Arrhenius and Eyring plots (where $r^2$ is 0.9995). The energy of activation ($E_a$) was $22.9 \pm 0.4$ kcal/mol; the enthalpy of activation ($\Delta H^{\dagger}$) was $22.3 \pm 0.4$ kcal/mol; and the entropy of activation ($\Delta S^{\dagger}$) was $5.4 \pm 1.3$ eu at 25°C. The small, positive $\Delta S^{\dagger}$ value is consistent with what has been observed for compounds which degrade by unimolecular mechanisms (Schaleger and Long, 1963).

The postulated, unimolecular, degradation mechanism for PD 144872 is shown in Scheme 1 where the reaction proceeds by an intramolecular process involving nucleophilic attack by the amino group at the $\beta$-carbon with expulsion of the bromide ion. This results in the formation of the corresponding aziridine or ring-closed product. Such a mechanism requires that the amino group exist in the nucleophilic neutral or free base form, consistent with enhanced stability in acidic media. Even though the first step of the reaction is shown to be reversible, in the absence of additional bromide ions, it can be treated as irreversible, and hence is the rate-limiting step for degradation of PD 144872. However, it should be noted that if additional bromide ions are added and the first step becomes truly reversible then factors which alter the rate of the aziridine ring opening step may affect the rate of degradation of PD 144872.

Subsequent to ring closure, nucleophilic attack by water or other suitable nucleophiles at an aziridine carbon atom results in ring opening and the formation of the corresponding aquated product. Comparable mechanisms have been used to describe the degradation of numerous 2-haloalkylamines (e.g., Cohen et al., 1948; Dermer and Ham, 1969) and several aliphatic and aromatic nitrogen mustards including melphalan (Valentin Feyns, 1984), mechlorethamine (Connors et al., 1986) and chlorambucil (Owen and Stewart, 1979; Ehrsson et al., 1980). Degradation of PD 144872 by a competing $S_N1$ mechanism, involving the formation of a rapidly hydrated primary carbonium ion, is extremely improbable due to the inability of the hydrogen atoms, attached to the halogenated carbon, to effectively stabilize a positive charge.

## Conclusions

The facile degradation of PD 144872 in aqueous solutions and in co-solvent systems and its good stability in the solid state suggest that the optimal parenteral dosage form for PD 144872 is a lyophilized one. Since no preservative is to be used in the formulation, an 8 h reconstitution shelf-life with less than 2% total degradation of PD 144872 was desired; therefore, a reconstitution pH of $\leq 3$ appears to be necessary. The reconstitution pH will be kept as close to 3 as possible to minimize pH-associated pain upon intravenous injection (Klement and Arndt, 1991). The maximal reconstitution concentration would be around 50 mg/ml at 25°C and about 30 mg/ml under refrigerated conditions.

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