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Degradation of berenil (diminazene aceturate) in acidic aqueous solution

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Abstract

The trypanocide berenil was assessed for chemical stability over the pH range 1–8 at 37°C and 0.2 M ionic strength. It was found to be sufficiently unstable under acid conditions that its therapeutic efficacy is most likely severely compromised when administered orally. At pH 3, the half-life was 35 min, decreasing to 1.5 min at pH 1.75. Reaction rate constants were corrected for the effects of buffer catalysis and were found to range from 2.00 min^{-1} at pH 1 to 6.1×10^{-6} min^{-1} at pH 8. The pH-rate profile displayed a region (pH 1–4) where specific acid catalysis was dominant, followed by a transitional region (pH 5–7), and finally a region (pH > 7) where uncatalysed degradation was most important. It is recommended that berenil be enteric coated for formulations to be used in treating Third World parasitic diseases.

Introduction

The parasites *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense* infect up to half a million humans in Africa per year leading to sleeping sickness (Bouteille et al 2003). The disease is characterized by two distinct stages, with stage 1 involving propagation through the haemolymphatic system and stage 2 affecting the central nervous system, resulting in death if left untreated. Current approved treatments for stage 1 infections are pentamidine via intramuscular injection for *T. b. gambiense*, and suramin via intravenous injection for *T. b. rhodesiense*. Treatments approved for stage 2 infections include melarsoprol (both parasites) and eflornithine (more effective against *T. b. gambiense*). There is an urgent need for an easily administered, affordable treatment for stage 1 trypanosomiasis for use in the developing nations of Africa, where the disease is most prevalent.

Berenil (diminazene aceturate) has been used successfully as a stage 1 trypanocide in the treatment of a variety of trypanosomiasis infections when administered intramuscularly in domestic livestock, including cattle, sheep, goats, pigs and horses (Peregrine & Mamman 1993). It is currently registered as a veterinary therapy but is not presently approved for use in humans (Bouteille et al 2003). Berenil has provided treatment in humans against both *T. b. gambiense* and *T. b. rhodesiense* when administered by intramuscular injection (Hutchinson & Watson 1962; Temu 1975; Abaru & Matovu 1981). Any observed side-effects of berenil were temporary and are no worse than those noted for suramin (Abaru et al 1984) or pentamidine (Bouteille et al 2003). In addition, the treatment period for berenil is shorter and the cost is lower than for the currently approved drugs.

The anti-trypanosomal activity of berenil has been observed after oral administration in humans (Bailey 1968); however, significant loss of berenil occurs within the stomach as a result of acid catalysed degradation of the drug molecule, thereby limiting the amount of active ingredient absorbed into the bloodstream. This was demonstrated using mice infected with *T. brucei*, where it was estimated that only $1/16^{th}$ to $1/66^{th}$ of the dose reached the jejunum after oral administration (Raether et al 1974). When administered directly into the jejunum, the effectiveness of berenil was similar to a subcutaneous injection, indicating that degradation of berenil occurs in the low pH region of the gastrointestinal tract.

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The berenil molecule contains two benzamidine moieties linked via a triazene at the 4 position of each ring. The triazene link is susceptible to cleavage resulting in the formation of 4-aminobenzamidine and a 4-amidinophenyldiazonium salt (Raether et al 1974). Acid catalysed degradation of other compounds containing a triazene link have also been observed producing analogous degradation products (Brown & Duffy 1967; Smith et al 1992). The diazonium salts are not directly detected, but are hydrolysed to the corresponding alcohols under aqueous conditions. Smith et al (1992) measured the decomposition rates of ten 1,3-dialkyl-3-acyltriazene analogues over the pH range 2–12 at 70°C using a constant lysine buffer concentration of 0.1 M. For each compound tested, the slope of the $\log_{10}k$ versus pH plots were approximately −1 in the linear portion of the curves. This is typical of acid catalysed decomposition following first-order kinetics (Connors et al 1986).

The degradation rates of compounds in solution can be dependent not only on pH, but also on the buffer concentration used to maintain solution acidity (Carstensen 1990). The influence of the buffer can be eliminated by measuring the rate constants at several buffer concentrations for each pH value. The reaction rate at zero buffer concentration (k_0) is then extrapolated from a plot of the observed rate constant versus buffer concentration. Several examples of this approach can be found in the literature (Notari & Caiola 1969; Hershfield & Schmir 1973; Ho & Fishbein 1994).

Currently, there is only one significant new drug under development (Swiss Tropical Institute, DB-289) for stage 1 *T. brucei* infections but this will not be available for several years until clinical trials are completed (Bouteille et al 2003). In the meantime, berenil could potentially be used as a treatment for stage 1 trypanosomiasis if a formulation could be developed to allow it to be administered orally. This would provide an easily administered, cheap and widely available drug source since large quantities are already manufactured for veterinary use. Berenil has the added advantages of a short treatment time and is effective against both types of *T. brucei* parasites encountered in Africa, removing the necessity of diagnosis between the *T. brucei* strains in patients.

The main purpose of the present study was to quantify the buffer independent rate constants for acid catalysed degradation of berenil using the "zero buffer" method. A further aim was to decide if formulation approaches could be used to limit degradation of the drug in the low pH regions of the gastrointestinal tract.

Materials and Methods

Materials

Berenil (diminazene aceturate) and 4-aminobenzamidine dihydrochloride were purchased from Sigma-Aldrich Chemical Company (Australia). 4-Hydroxybenzamidine hydrochloride was purchased from Toronto Research Chemicals Inc. (Canada). Water was purified using a Milli-Q Gradient A10 purifier system (Millipore Australia Pty. Ltd). All other chemicals were of analytical grade.

Buffer solutions

Solutions at pH 1 to 2 were prepared with hydrochloric acid, pH 3 and 4 with formic acid/ammonium formate, pH 5 with acetic acid/ammonium acetate, and pH 6 to 8 with sodium dihydrogen orthophosphate/disodium monohydrogen phosphate. The ionic strength of all solutions was adjusted to 0.2 M using NaCl. Three different buffer concentrations were used to prepare the pH 3–8 solutions in order to determine the buffer-independent hydrolysis rate constants (k_0). The maximum buffer concentration employed was dependent on the ionic strength attributed to the buffer components so as not to exceed an overall ionic strength of 0.2 M. The buffer concentrations chosen were 10, 50 and 100 mM (pH 3–6), 10, 50 and 85 mM (pH 7) and 10, 35 and 65 mM (pH 8), and the buffer compositions are shown in Table 1.

A Radiometer PHM240 3-figure pH meter (Radiometer, Copenhagen, Denmark), a semi-micro glass pH electrode and a temperature probe (automatic temperature compensation) were used to determine solution pH. Calibration of this system was performed using the IUPAC buffer solutions (Radiometer) 9.180, 6.865, 4.005 and 1.679. A 2-point calibration was used before sample measurement using buffer solutions of the narrowest range that spanned the expected sample pH. All buffer pH values were recorded at 37°C when prepared and after each experiment. In all cases, the pH change was less than 0.05 during the course of the study, indicating that the buffer capacity was sufficient.

Berenil degradation experiments

The pre-warmed (37°C) buffer solutions (10 mL) were pipetted into 12-mL polypropylene tubes containing preweighed berenil. The final sample concentrations were 0.2 mg mL^{-1} (pH 1–2), 0.5 mg mL^{-1} (pH 3–5) and 0.02 mg mL^{-1} (pH 6–8). The solutions were then vortexed at high speed for 10 s (pH 1–2) or 30 s (pH 3–8) and placed in a shaking water bath set at 37°C and 35 rev min^{-1}. Aliquots of the reaction mixtures at pH 1–5 (0.1 mL) were taken at appropriate intervals, quenched with phosphate buffer (0.9 mL, 100 mM, pH 7) and placed in the high-performance liquid chromatography (HPLC) sample compartment at 5°C before analysis. The pH 6–8 samples were not quenched, but frozen immediately after each time point and analysed as a batch after the sampling period was completed. Experiments for the pH 1–6 samples were conducted for sufficient time to observe approximately 90% degradation of the material, with samples taken at time points established from preliminary experiments to ensure adequate data points during the course of each experiment. At least eight data points were analysed over the period of each experiment. A 20-day sampling period for the pH 7–8 samples was conducted, resulting in approximately 20% degradation of the initial berenil mass.

The initial time point assay results from the berenil pH 8 samples were used as a control to ensure the freezing process did not affect the pH 6–8 samples during storage.

Table 1 Experimental conditions, rate constants and half-lives observed for the degradation of berenil. The variation in pH measurements quoted reflects the change in pH over the course of the experiments

Observed pH at 37°C	Buffer composition			Observed rate constant (k_{obs}, min^{-1}) (s.e.)	"Zero buffer" rate constant (k_0, min^{-1}) (s.e.)	$t_{1/2}$ (min) (s.e.)
	[Acid]	[Anion]	[NaCl]			
1.03 ± 0.03	0.100	—	0.100	2.00 (0.073)	—	0.35 (0.01)
1.54 ± 0.03	0.0316	—	0.168	0.868 (0.042)	—	0.80 (0.04)
1.75 ± 0.04	0.0178	—	0.182	0.475 (0.025)	—	1.46 (0.07)
1.98 ± 0.04	0.0100	—	0.190	0.201 (0.004)	—	3.46 (0.07)
2.93 ± 0.03	0.00803	0.00197	0.198	0.0197	0.0199 (0.003)	34.9 (4.6)
3.03 ± 0.02	0.0401	0.00990	0.190	0.0283		
3.02 ± 0.02	0.0803	0.0197	0.180	0.0309		
4.04 ± 0.03	0.00289	0.00711	0.193	2.72×10^{-3}	2.7×10^{-3} (3×10^{-4})	2.6×10^{2} (3×10^{1})
4.02 ± 0.03	0.0145	0.0356	0.164	3.54×10^{-3}		
3.95 ± 0.02	0.0289	0.0711	0.129	3.91×10^{-3}		
4.93 ± 0.03	0.00289	0.00711	0.193	3.93×10^{-4}	3.92×10^{-4} (7×10^{-6})	1.77×10^{3} (3×10^{1})
4.95 ± 0.02	0.0145	0.0356	0.164	3.74×10^{-4}		
4.99 ± 0.03	0.0289	0.0711	0.129	3.69×10^{-4}		
5.93 ± 0.03	0.00849	0.00151	0.189	5.18×10^{-5}	5.07×10^{-5} (7×10^{-7})	1.37×10^{4} (2×10^{2})
5.96 ± 0.03	0.0425	0.00755	0.170	5.83×10^{-5}		
5.98 ± 0.03	0.0849	0.0151	0.070	6.46×10^{-5}		
6.98 ± 0.03	0.00360	0.00640	0.187	9.49×10^{-6}	9.1×10^{-6} (4×10^{-7})	7.6×10^{4} (3×10^{3})
7.01 ± 0.02	0.0180	0.0320	0.134	10.0×10^{-6}		
7.02 ± 0.02	0.0306	0.0544	0.006	11.4×10^{-6}		
7.99 ± 0.02	0.000532	0.00947	0.171	7.28×10^{-6}	6.1×10^{-6} (9×10^{-7})	1.14×10^{5} (1×10^{4})
7.98 ± 0.02	0.00186	0.0331	0.099	8.35×10^{-6}		
7.99 ± 0.02	0.00346	0.0616	0.012	1.18×10^{-5}		

The use of this assay result was justified since the amount of berenil degradation at pH 8 at the initial time point is negligible.

HPLC method

The berenil degradation samples were analysed using a Waters Alliance HPLC system containing a Phenomenex Synergi Hydro (2.0×50 mm, 4 μm) column at 25°C. The mobile phase buffers used were Milli-Q water (solvent A), acetonitrile (solvent B), 2 M ammonium formate (solvent C) and 1% aqueous formic acid (solvent D), with a total flow of 0.4 mL min^{-1}. The injection volume was 10 μL. Isocratic conditions (96% A, 0% B, 2% C, 2% D) were maintained for 1 min, followed by a gradient of 5.7% B min^{-1} for 10 min before returning to initial conditions. The percentages of solvents C and D were kept constant throughout the experiment. The sample compartment temperature was maintained at 5°C and the detection wavelength was 254 nm. The method used led to the elution of berenil in approximately 6 min and the two observed degradants in 1.5 and 1.8 min.

Liquid chromatography–mass spectrometry (LC-MS) method

The HPLC system described above was coupled to a Waters ZQ mass spectrometer operating under positive ion electrospray ionization conditions that had been optimized for the detection of berenil. General instrument conditions were as follows: capillary voltage 3.2 kV, cone voltage 20 V, desolvation temperature 350°C and desolvation gas flow 400 L h^{-1}. Under these conditions, mass spectra were acquired for berenil and the two major degradation products.

Determination of solution components during degradation

Standard plots were prepared for berenil (10–150% nominal concentration) and 4-aminobenzamidine dihydrochloride (15–150% expected formation). The regression results were then used to determine concentration values of the known components of the degradation samples. All calculations were based on the free base forms of berenil and 4-aminobenzamidine. HPLC spiking experiments confirmed the identity of all of the components in the degradation samples.

Calculation of degradation rate constants

The plot of $\log_{10}$(% berenil remaining) versus time provided linear plots for all experiments, where the slope of the line was used to calculate the observed rate constant k_{obs} as follows:

$$k_{obs} = -(\text{slope} \times \ln 10) \quad (1)$$

where $\ln 10 = 2.303$.

The validity of the linear plots was tested using simple analysis of variance methods based on Student's *t*-test and Fisher's overall *F* ratio (Draper & Smith 1981).

The value of the buffer independent rate constant, k_0, was calculated from regression analysis of each plot of the observed rate constants ($k_{buffer\ 1}$, $k_{buffer\ 2}$ and $k_{buffer\ 3}$) versus the buffer concentration (pH 3–8). The values for k_0 (pH 3–8) and k_{obs} (pH 1–2, considered as k_0 owing to the absence of buffer salts) at each pH were subsequently used to prepare a pH-rate profile and calculate degradation half-lives:

$$t_{1/2} = \ln 2/k_0 \quad (2)$$

where $\ln 2 = 0.693$.

Results and Discussion

A typical HPLC chromatogram acquired during the acid catalysed degradation of berenil is shown in Figure 1. The expected identity of degradant 1 (retention time = 1.5 min) was confirmed as 4-aminobenzamidine by LC-MS analysis ($MH^+ = 136$) and HPLC spiking experiments with an authentic sample. Degradant 2 (retention time = 1.8 min) was identified after the collection of berenil degradation data at each pH value was investigated. This degradant was confirmed as 4-hydroxybenzamidine by LC-MS analysis ($MH^+ = 137$) and HPLC spiking experiments with 4-hydroxybenzamidine hydrochloride. The identification of these two major degradation products supports the proposed mechanism of berenil degradation depicted in Figure 2.

The analytical method used to quantify berenil and 4-aminobenzamidine demonstrated good stability and validation criteria were within acceptable limits. The calibration plots for both compounds demonstrated a linear response over the concentration range of interest, with R^2 values greater than 0.999 and consistent slopes and y-intercepts over a 4-week period, for multiple sample and standard preparations. Student's *t* values (two-tailed), calculated from the standard errors of the linear regression slopes, corresponded to *P* values for significance that never exceeded 0.0001, while the intercepts were never significantly different to zero. Overall Fisher *F* ratios for the linear regressions showed they were highly significant, with *P* values always less than 0.00002. The slope and intercept achieved for berenil were $(6.2 \pm 0.3) \times 10^7$ abs mg^{-1} mL^{-1} and $-25\,000 \pm 25\,000$ abs; for 4-aminobenzamidine they were $(4.0 \pm 0.1) \times 10^7$ abs mg^{-1} mL^{-1} and 3000 ± 4000 abs, respectively. Complete recovery of berenil was observed from the control samples for all experiments, which validated the quenching process for experiments in the pH 1–5 range and the storage procedure for the pH 6–8 samples.

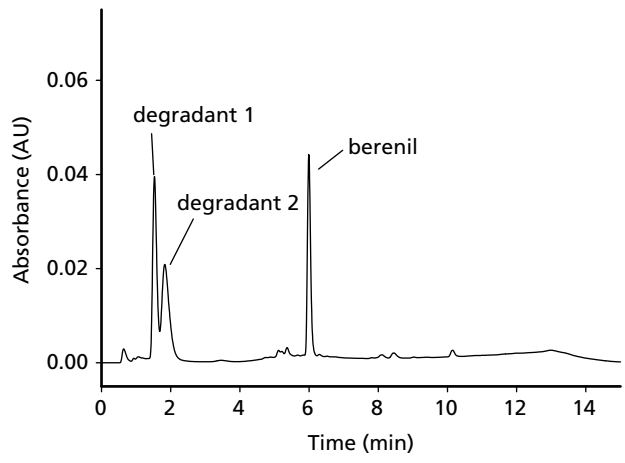

Figure 1 Chromatogram of a berenil sample after treatment for 55 min at 37°C in buffer (pH 3, 50 mM). The two early eluting components correspond to the major degradants depicted in Figure 3.

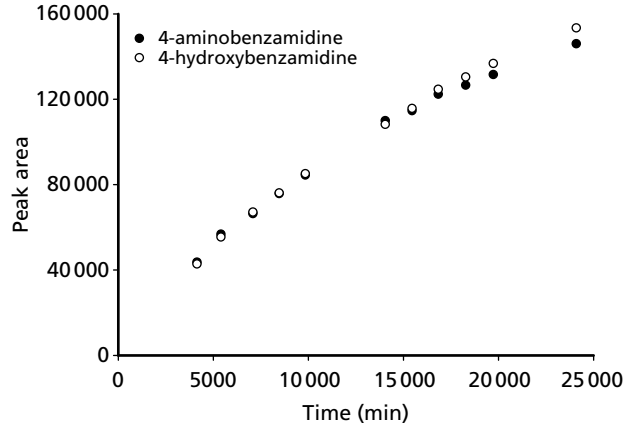

Figure 2 Plot of the formation profiles of 4-aminobenzamidine and 4-hydroxybenzamidine at pH 6, 10 mM total buffer concentration.

The measured concentration of 4-aminobenzamidine at each time point demonstrated mass balance based on the measured concentration of berenil remaining in solution. Additionally, the peak areas observed for both 4-aminobenzamidine and 4-hydroxybenzamidine throughout the sampling periods were approximately equal, as expected for compounds of similar molecular structure and size. An example of this is shown in Figure 2 for the pH 6 degradation experiment (10 mM buffer), where measured peak areas for the two degradants were found to be equal over the course of the experiment. This confirms the acid catalysed degradation of berenil occurs via scission of the triazene linkage resulting in the formation of two fragments. 4-Hydroxybenzamidine was most likely produced via rapid hydrolysis of the initial scission fragment 4-amidinophenyldiazonium salt, which was not directly detected during HPLC or LC-MS analyses. This complete degradation pathway is in agreement with the proposed pathway shown in Figure 3 and with the aqueous acid degradation studies of other triazene analogues (Brown & Duffy 1967; Smith et al 1992).

The rate constants for the degradation of berenil were measured over a range of pH values (pH 1–8). Experiments for the pH range 1–2 were performed in HCl solutions (ionic strength adjusted with sodium chloride). Buffers for the pH range of 3–8 were prepared using appropriate buffer systems (Table 1). Three buffer concentrations were used to correct for catalytic effects. Typical profiles of berenil

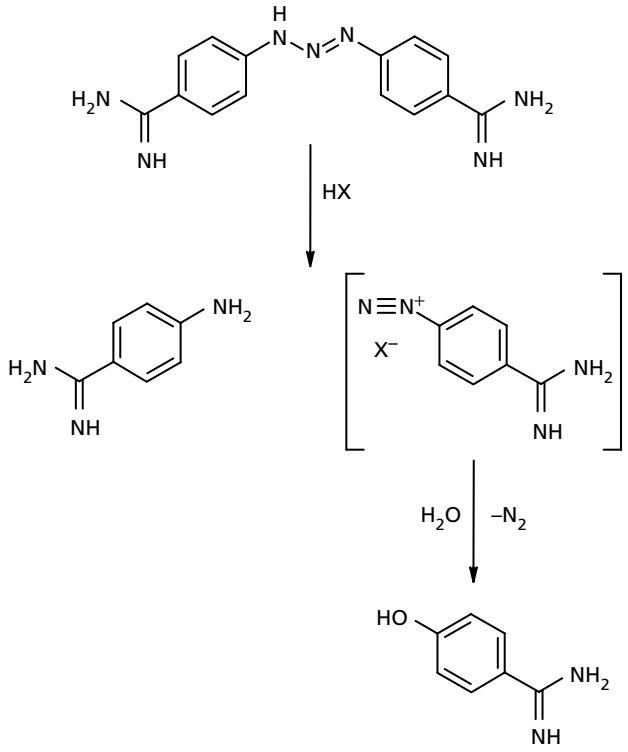

Figure 3 Acid catalysed degradation pathway of berenil. The diazonium species depicted is not isolated but is implied by detection of the 4-hydroxybenzamidine. The identity of both of the degradation products was confirmed by LC-MS studies and HPLC spiking experiments.

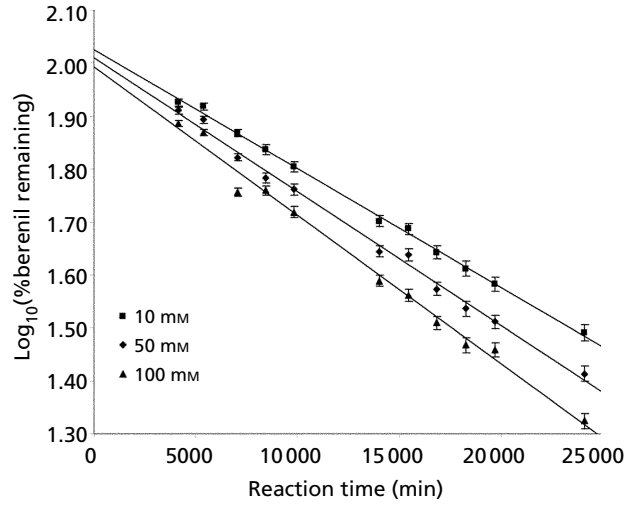

Figure 4 Plot of the degradation profile of berenil at pH 6 under three buffer conditions. The error bars represent ± 1 s.e. The relationship between degradation rate and buffer concentration is demonstrated by this plot.

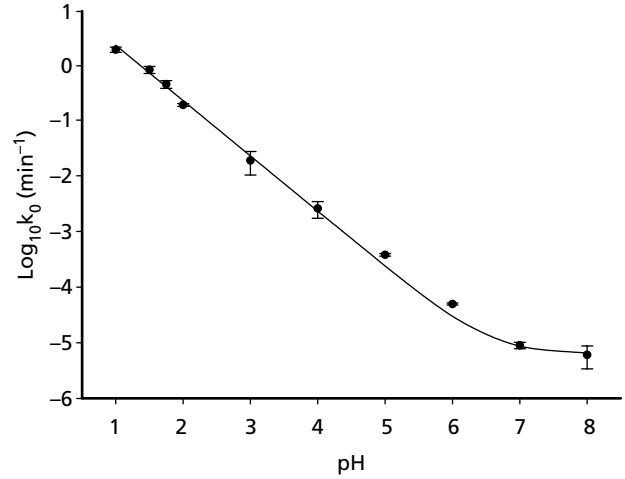

Figure 5 Plot of $\log_{10}$ of the rate constants for berenil degradation against pH. The line represents the non-linear regression model fitted to the data. The error bars represent three standard errors, calculated for the individual pH-rate experiments.

degradation using three buffer concentrations are shown in Figure 4. The pseudo-first-order k_{obs} values from the slopes of these plots were then plotted against buffer concentration and k_0 calculated by extrapolation of the slope to the y-axis. For example, at pH 6, the *P* value calculated from the *t* value for the slope was 0.049, while the *P* value from the overall *F* ratio was 0.055. These demonstrate a linear relationship at the 95% confidence interval. The observed rate constants calculated from each solution (pH 1–8) and their standard deviations are shown in Table 1. The dependence of buffer concentration on the reaction kinetics is shown by comparing the k_{obs} values at 100 mM buffer concentration with the extrapolated k_0 values. For a given solution pH, a difference of up to 2-fold was observed between k_{obs} and k_0.

Specific acid catalysis of the berenil degradation under aqueous conditions was demonstrated from the plot of $\log_{10}k_{obs}$ versus pH (Figure 5). A linear relationship ($R^2 = 0.996$) was observed over the pH range 1–4, with a slope of −0.99. The slope of the line over the pH range 4–7 also indicated an acid catalysed degradation, although the deviation from the ideal slope suggested that the mechanism is a combination of acid mediated and uncatalysed hydrolysis. Obviously, as the pH approached neutrality, the uncatalysed reaction became dominant, leading to the non-linear portion of the $\log_{10}k_{obs}$ versus pH plot (Figure 5).

The plot of $\log_{10}k_{obs}$ versus pH can be modelled as an example of Equation (3), where k_H represents the acid catalysed rate constant and k_{H2O} represents the uncatalysed rate constant.

$$k_{obs} = k_H[H^+] + k_{H2O} \quad (3)$$

Non-linear regression analysis of the raw data resulted in values for the rate constants as:

$k_H = 23.99\ \text{min}^{-1}\ \text{M}^{-1}$

$k_{H2O} = 6.35 \times 10^{-6}\ \text{min}^{-1}$

The non-linear regression analysis of the raw data is depicted on the plot of $\log_{10}k_{obs}$ versus pH (Figure 5), where the fitted line represents the model derived from Equation 3 and the rate constant values shown above.

The reaction half-life ($t_{\frac{1}{2}}$) values shown in Table 1 clearly demonstrate the likely formulation problem associated with the oral administration of berenil as fasting gastric pH conditions in humans are typically over the range 1.5–3.5 (Marieb 1998).

Conclusions

Berenil was found to undergo degradation via hydrolysis over the pH range 1–8. The degradation rate, after correction for buffer effects, was found to be dependent not only on specific acid catalysis, but also on an uncatalysed hydrolysis mechanism. Over the pH range 1–4, the effect of the uncatalysed reaction mechanism was negligible, leading to a classic $\log_{10}k_{obs}$ versus pH plot with a slope of -1. Over the pH range 4–8, the uncatalysed reaction became progressively more significant, producing a deviation from the linear profile, which reached a plateau at pH values greater than 7.

Non-linear regression analysis of the raw data was able to fit the data to Equation 3, which accounts for the acid catalysed rate constant k_H and the uncatalysed rate constant k_{H2O}. The degradation products formed were identified as 4-aminobenzamidine and 4-hydroxybenzamidine, the latter most likely produced via rapid hydrolysis of the initial degradation product, 4-amidinophenyldiazonium salt.

The measured half-life of berenil at a pH of 3 was approximately 35 ± 5 min, in accord with previous studies showing that berenil was unlikely to be sufficiently stable under gastric conditions to enable oral administration. Berenil appears a likely candidate for development of an enteric coated formulation, with the aim of releasing drug in the near neutral sections of the gastrointestinal tract. If this approach were successful, orally administered berenil could be used as the treatment of choice for stage 1 trypanosomiasis infections.

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