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RESEARCH ARTICLE

Pre-formulation and chemical stability studies of penethamate, a benzylpenicillin ester prodrug, in aqueous vehicles

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Abstract

Penethamate (PNT) is a diethylaminoethyl ester prodrug of benzylpenicillin used to treat bovine mastitis via the intramuscular route. Because of its instability, PNT products must be reconstituted before administration and the reconstituted injection has a short shelf life (7 days at 2–8°C). The purpose of this paper was to investigate whether the stability of PNT can be improved in order to achieve a chemically stable ready-to-use aqueous-based PNT formulation or at least to extend the shelf life of the reconstituted suspension. A chemical stability study of PNT in aqueous-based solutions as a function of pH, buffer strength, solvent mixtures and temperature, supported by studies of its solubility in mixed solvents, allowed predictions of the shelf life of PNT solution and suspension formulations. PNT degraded in aqueous solutions by several pathways over the pH range 2.0–9.3 with a V-shaped pH-rate profile and a minimum pH of around 4.5. The stability of PNT solutions in mixed solvents was greater than in aqueous solutions. For example, in propylene glycol:citrate buffer (60:40, v/v, pH 4.5), the half-life of PNT was 4.3 days compared with 1.8 days in aqueous buffer. However, solubility of PNT in the mixed solvent was higher than that in aqueous solution and this had an adverse effect on the stability of suspensions. By judicious choosing of pH and mixed solvent, it is possible to achieve a storage life of a PNT suspension of 5.5 months at 5°C, not sufficient for a ready-to-use product but a dramatic improvement in the storage life of the reconstituted product.

Keywords: Penethamate, prodrug, benzylpenicillin, degradation kinetics, chemical stability, aqueous vehicle, ready-to-use formulation

Introduction

Bovine mastitis is treated with antibiotics often via the intramuscular route whereby penethamate (PNT), a diethylaminoethyl ester prodrug of benzylpenicillin (BP), is commonly used due to its advantageous physicochemical properties compared with BP and other β-lactam antibiotics. On intramuscular administration, PNT (pK_a 8.4, liphophilic weak base) circulates in the blood with an estimated 10% in the non-ionized form (BP only <0.001%). It is postulated to readily pass the blood–milk barrier[1–3] becoming trapped in the milk (pH 6.4–6.6) where the majority of PNT is ionized (~99%). In addition, PNT is hydrolysed to BP which is highly ionized in the milk thereby limiting its return to the circulation and resulting in accumulation of BP in the udder[4]. As the milk to plasma ratio is much higher for PNT, being in the range of 2 to 6.1[5,6], compared with other β-lactam antibiotics such as BP (0.2), amoxicillin (0.26) or ampicillin (0.26[6], PNT is the preferred β-lactam antibiotic for treating bovine mastitis intramuscularly.

Currently, PNT is only available in the market in the form of a powder for reconstitution in a buffer prior to intramuscular injection (products: Mamyzin-Boehringer Ingelheim, United Kingdom or Penethaject-Bomac Laboratories Ltd., New Zealand). These reconstituted PNT suspensions (~333 mg mL^{-1}) have a limited chemical shelf life of 7 days at 2–8°C or 2 days at 15–20°C. From the veterinarian's perspective, the reconstitution step is

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inconvenient in the field and therefore a ready-to-use aqueous-based PNT product is sought as a preferred formulation type if available. As PNT, like other β-lactam antibiotics, is susceptible to degradation involving various nucleophiles, acid base reagents, metal ions, oxidizing agents and solvents[7–9], a systematic investigation of the kinetics of PNT in aqueous-based vehicles is required. Due to the recent development of a high-performance liquid chromatography (HPLC) assay for PNT in aqueous solutions[10], it is now for the first time possible to determine the chemical stability of the PNT itself.

There are only a few old reports in the literature on the chemical stability of PNT[11–14]. However, in all these studies, the measurements were based on UV spectrophotometric analysis or biological assays of BP, the hydrolytic product, rather than PNT itself. Jensen et al.[13] reported the half-life ($t_{1/2}$) of PNT as 23 min and 220 min at pH 7.3 and pH 6.2, respectively, at 37°C. Similarly, Dinsmore and Bailey[11] determined the rates of hydrolysis of PNT in solutions at pH 2–7.5 and reported that PNT is most stable at pH 4.9, with <10% hydrolysed after 3 h at 25°C. Improved stability of PNT was reported in suspension, with 7% PNT hydrolysed at pH 6 after 7 days at 25°C[14]. Hallas-Moller et al.[12] studied the degradation of PNT in plasma samples at different pH and temperatures and reported that more than 85% of PNT degraded within 1 h at 37°C. In these cases, the investigators assumed, first, that the PNT was converted only to BP, which is not the case given that PNT can follow alternative degradation pathways[10], second, that the BP was stable over the period of the kinetic studies and, third, that the PNT did not interfere with the spectrophotometric analysis of BP. If the PNT degrades via additional pathways, rather than just to BP, the chemical degradation will have been underestimated by studies based on BP analysis. In addition, if BP degrades during a kinetic study, the stability assessment based on the spectrophotometric assay of BP will again underestimate the degradation of PNT. Given these uncertainties, an investigation of the stability of PNT, using a stability-indicating assay for PNT itself and for BP, is needed for the efficient and reliable development of a novel ready-to-use aqueous-based PNT product.

This paper addressed the research question of whether the hydrolysis of PNT can be minimized during shelf life (t_{90}) in order to achieve an acceptable chemical stability of a ready-to-use aqueous-based PNT formulation or at least extend the storage life of the reconstituted injection. This initially required a fundamental stability study of PNT in aqueous-based solutions as a function of pH, buffer strength, solvent mixtures and temperature using a stability-indicating assay for PNT and BP but the entire degradation pathways of PNT was not part of this investigation. Solubility studies in mixed solvent systems were also conducted enabling estimations of shelf-life stability of PNT suspensions in mixed solvent formulations.

Experimental

Chemicals

Penethamate hydriodide (batch no. PE-0808001, purity 98.5%) was kindly donated by Bioquim, SA, Barcelona, Spain. Benzylpenicillin potassium (batch no. 054K27561) was purchased from Sigma Aldrich, MO. Methanol, acetonitrile (HPLC grade) and propylene glycol (PG), polyethylene glycol (PEG 400) and ethanol (analytical grade) were obtained from Merck, Germany. Milli-Q water (Millipore Corporation, Bedford, MA) was used for mobile phase and sample preparations. All other chemicals and solvents were analytical grade (BDH Chemicals Ltd, England and Ajax Finechem New Zealand).

Instrumentation and chromatographic conditions

The stability-indicating HPLC assay method for PNT used in this study has been described previously[10]. Briefly, the HPLC system comprised a Shimadzu LC-20 Prominence series (Shimadzu, Kyoto, Japan) LC system equipped with an LC-20AD quaternary pump, an SPD-M20A diode array detector (DAD) and SIL-20AC auto-injector set at 4°C. The output signal was monitored and processed using Class VP 7.4 software (Shimadzu, Kyoto, Japan). The chromatographic separation was achieved on a Luna C_{18}, 150 mm × 4.6 mm i.d., 5 μm particle size column (Phenomenex, New Zealand). The isocratic elution at a flow rate of 1 mL min^{-1} was achieved using a methanol: acetonitrile: 50 mM acetate buffer pH 5.0 (35:5:60, v/v/v) as mobile phase. The injection volume was 100 μL and the wavelength for detection was 230 nm. A quenching solution[10] containing PG-acetate buffer (1 M; 50:50, v/v; pH 4.8) was used to limit the degradation to negligible levels before and during analyses. The retention times for PNT and BP were 10.3 and 7.3 min, respectively. The assay of PNT was stability indicating with adequate precision (RSD ≤ 1%) and accuracy (98–99%) and the limit of quantification of PNT and BP was 0.09 μg mL^{-1} and 0.42 μg mL^{-1}, respectively.

Determination of apparent pK_a by UV absorbance spectroscopy

The pK_a (8.4 or 8.5) of PNT is cited in various reports[3,4,15,16]. None of these reports, however, provides details of methodology used for the determination of pK_a of PNT. Given its instability, it is difficult to determine the pK_a because of significant degradation during the required measurements. As pK_a is an important parameter in the stability and pharmacokinetics of PNT, an effort was made to confirm the previous reports on pK_a of PNT.

Absorbance spectra of freshly (<1–2 min) prepared PNT aqueous solutions over the pH range 3.8–10.2 were measured using a Shimadzu UV/VIS Spectrophotometer (UV 1601/PC, Japan) to determine the absorption maxima of the ionized and non-ionized species. An analytical wavelength of 250 nm was selected to determine the pK_a based on the method described by Albert and Serjeant[17]. Briefly, a series of buffers of low ionic strength ($\mu = 0.01$)

was prepared as described by Perrin[18]. Then, buffer (2.95 mL) was mixed with 50 μL of fresh PNT stock solution in methanol (7 mg mL^{-1}) to give a final drug strength of 116 μg mL^{-1} (2.6×10^{-4} M), and a final methanol concentration of 1.6%. The absorbance was measured at 23°C at known times for up to 4 min after mixing of the buffer and stock solution. The absorbance at time zero (A_0) was obtained by extrapolation of the linear least squares regression line of absorbance versus time to time zero. Equation 1 was fitted by non-linear least squares regression to estimate A_u, A_i and pK_a using PRISM software (GraphPad version 5.0).

$$A_0 = \frac{A_u + A_i \times 10^{(pK_a - pH)}}{1 + 10^{(pK_a - pH)}} \qquad (1)$$

where A_0 is the absorbance of the solution at time zero obtained by extrapolation, A_u is the absorbance of unionized species and A_i the absorbance of the ionized species. Duplicate experiments were conducted and the mean values were used for calculation of pK_a.

Solubility studies of PNT in cosolvent systems

In order to predict the chemical stability of PNT in suspensions in cosolvent systems, the solubilities of PNT in cosolvent PG (5, 20, 40, 60% v/v)—citrate buffer (50 mM, pH 4.5) systems were determined at 30°C. In addition, solubility of PNT in cosolvent PG (5% v/v)—citrate buffers (50 mM, pH 4.5) was determined at 5°C and 20°C. Excess PNT (80 mg mL^{-1}) was added to the solvent (4 mL) in screw-capped glass tubes and the tubes were shaken (100 opm) at 5°C or 20°C or 30°C in a shaking water bath for 2 h during which PNT showed negligible degradation. Samples were centrifuged at 3000 rpm (Eppendorf centrifuge, Model 5810 R, Germany) for 10 min, and supernatants were then filtered (0.22 μm, Millipore) using a warm (30°C) syringe to avoid precipitation, diluted with quenching solution and then analyzed for PNT by HPLC. All solubility experiments were performed in duplicate.

Chemical stability studies of PNT

Degradation kinetics

The kinetic studies were carried out as follows: buffer (9.5 mL) in a 25 mL stoppered Erlenmeyer flask was brought to water bath temperature (30 ± 0.1°C) and then 0.5 mL of stock solution of PNT (10 mg mL^{-1} in PG) was added with mixing. At appropriate times, samples (0.5 mL) of the reaction mixture were taken and added to 4.5 mL of quenching solution (at 0°C, slushy ice bath) and analyzed by HPLC within 24 h. Chemical stability was monitored over 3–4 half-lives of PNT. Samples were protected from light during the study and experiments were performed in duplicate.

pH-rate profile

Buffer solutions (50 mM) of phosphate (pH 2.0–3.0 and 6.5–8.0), acetate (pH 4.0–6.0) and borate (pH 9.0–9.3) were adjusted to constant ionic strength ($\mu = 0.15$) with sodium chloride. The pH of each reaction mixture was measured (Sartorius basic pH meter PB-11, Germany) at the beginning and end of data collection and no measurable change in pH was observed.

Buffer

PNT (500 μg mL^{-1}) solutions in acetate (pH 4.52 and 6.01), phosphate (pH 3.01, 6.01, 7.15 and 8.04) and citrate buffers (pH 4.52) were prepared and compared at two buffer concentrations (50 mM and 150 mM) with constant ionic strength ($\mu = 0.5$) adjusted with sodium chloride at 30 ± 0.1°C to investigate the catalytic effect of buffer on the degradation of PNT.

Ionic strength

PNT (500 μg mL^{-1}) in buffer (50 mM) solutions of phosphate (pH 3.01) and borate (pH 9.01) buffer with varying ionic strength ($\mu = 0.05$, 0.15 and 0.5) were investigated for the salt effect on the stability of PNT at 30 ± 0.1°C. The modified Debye-Huckel equation (Equation 2) recommended for solutions with higher values of ionic strength ($\mu = 0.5$) was used[19]:

$$\log k_{obs} = \alpha + 2.Q.Z_A Z_B.\frac{\sqrt{\mu}}{\left(1 + \sqrt{\mu}\right)} \qquad (2)$$

where k_{obs} is the observed rate constant, α is the log of the rate constant at zero ionic strength, Q is a constant for the solvent at a given temperature ($2Q = 1.026$ at 30°C[19]) and Z_A and Z_B are the charges on reactants A and B, respectively. A plot of log k_{obs} versus $\sqrt{\mu} / (1 + \sqrt{\mu})$ was used to evaluate the effect of μ on the degradation rate of PNT.

Temperature

Solutions of PNT (500 μg mL^{-1}) were prepared in phosphate (pH 3.01 and 8.04) and acetate (pH 6.01) buffers (50 mM) and ionic strength (0.15). Degradation was observed at 30 ± 0.1°C, 40 ± 0.1°C and 50 ± 0.1°C. The observed pseudo-first-order degradation rate constant (k_{obs}) for each buffer/temperature condition was determined by non-linear least squares regression. The energy of activation (E_a) for PNT degradation in each buffer was determined by non-linear least squares regression using Equation 3.

$$k_{obs} = A.e^{\frac{-E_a}{RT}} \qquad (3)$$

where R is the universal gas constant and T is the absolute temperature (K) and A the frequency factor.

Cosolvent systems

In preliminary studies with cosolvents reported earlier[10], PG:buffer (pH 4.5, 1 M; 50:50 v/v) was found to be most promising for the stability of PNT. Further studies on the stability of PNT (500 μg mL^{-1}) were carried out in various PG:citrate buffer (50 mM) cosolvent systems (20:80, 40:60 and 60:40 v/v) at pH 3.01, 4.52 and 6.01 at 30 ± 0.1°C following a procedure similar to that for degradation kinetics studies (see section on Chemical Stability Studies of PNT).

Results

Ionization constant (pK_a) for PNT

Determination of pK_a is a challenge for rapidly degrading molecules like PNT in aqueous solutions. In this study, absorbances of the PNT solutions changed over 4 min, changes being more rapid at higher pH values. Based on these changes, it is estimated that 5% degradation occurred at pH 8 indicating that extrapolation to time zero was necessary for the accurate determination of pK_a. The pK_a of PNT determined using Equation 1 was 8.40 ± 0.13 (mean ± SD) consistent with earlier reports[3,4,15,16].

Solubility of PNT in cosolvent systems

PG increased the solubility of PNT up to three times at a temperature of 30°C (Table 1), the log solubility being linearly related to the mole fraction of PG in the PG:citrate buffer solvent systems (Figure 1). The dielectric constants of the mixtures (Table 1) were calculated from the relation $_{mix} = {}_{w} f_w + {}_{PG} f_{PG}$[20], where ε and f are the dielectric constant and volume fraction, respectively, and the subscripts *mix*, *w* and *PG* refer to the mixed solvent, water (buffer) and PG, respectively. A linear relationship was observed between log solubility and dielectric constants of the mixtures.

Chemical stability of PNT

Degradation kinetics

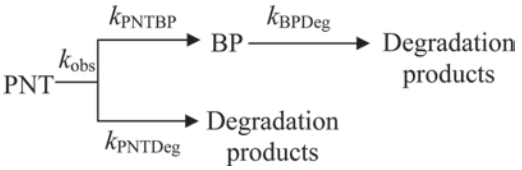
In addition to the PNT peak, BP and other degradation peaks were observed in the chromatograms but the other degradation peaks were not identified. Various kinetic models (first order and zero order) were fitted to the PNT-time data from each kinetic experiment by non-linear regression using PRISM (Graph pad *ver* 5.0) software and the best-fit model selected based on the Akaike's information criterion (AIC). On this basis, the first-order model gave the best fit (Figure 2), indicating pseudo-first-order degradation kinetics for PNT in aqueous solutions ($R^2 \geq 0.99$).

Figure 3A and 3B shows the degradation of PNT and subsequent formation of BP over time in acidic and alkaline pH at 30°C, respectively. It is apparent that the sum of molar concentrations of PNT and BP is not constant, the amount of BP found at low pH is particularly small (Figure 3A) and BP also degrades over time (Figure 3B). These facts and the presence of other degradation peaks indicate that PNT does not just degrade to BP and that the BP produced, also degrades (Scheme I), but that the contributions of the degradation pathways are dependent on the pH of the solution.

Table 1. Solubility and stability of PNT in PG-citrate buffer mixtures (pH 4.5, 50 mM) at 30°C.

PG (% v/v)	Dielectric constant (ε)	Solubility of PNT (mg mL^{-1})
5	77.6	8.9
20	70.0	11.6
40	60.8	18.8
60	51.2	28.2

PNT, penethamate; PG, propylene glycol.



where k_{obs} is the observed rate constant which is the sum of k_{PNTBP} and k_{PNTDeg}. As only PNT and BP were quantified, it is possible that k_{PNTDeg} represents several other parallel degradation pathways of PNT. At each pH, data for PNT and BP were regressed simultaneously on time (Equations 4 and 5) in order to estimate k_{PNTBP}, k_{PNTDeg} and k_{BPDeg}.

$$P = P_0\ e^{(-(k_{PNTBP}+k_{PNTDeg})*t)} \tag{4}$$

$$B = B_0 e^{-k_{BPDeg}*t} + \frac{k_{PNTBP} P_0}{(k_{BPDeg} - k_{PNTBP} - k_{PNTDeg})} \left[e^{-(k_{PNTBP}+k_{PNTDeg})*t} - e^{-k_{BPDeg}*t} \right] \tag{5}$$

where B and P represent BP and PNT concentrations at time t, B_0 and P_0 are the initial concentration of BP and PNT, respectively. At pH 2, 3 and 4 where no or very little BP was found (Figure 3A), the model (Equation 5) was collapsed to fit for k_{PNTDeg} only. At pH 7.5, 8, 9 (Figure 3B) and 9.3 where PNT degraded mainly to BP, the model was fitted to calculate k_{PNTBP} and k_{BPDeg}. At intermediate pH (4.5–7.0), degradation of PNT was described by all reactions according to Scheme I. The best-fit model was selected based on AIC. Table 2 shows the pH-rate profiles for the rate constants involved in various degradation reactions of PNT according to Scheme I. The degradation

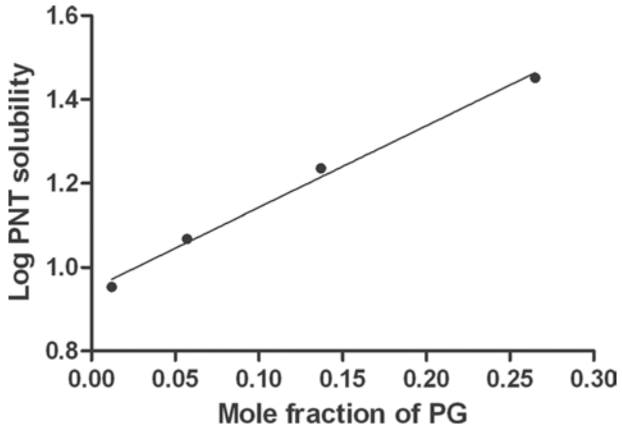


Figure 1. Effect of propylene glycol (PG) content on the solubility of penethamate (PNT) in PG:citrate buffer (pH 4.5) mixed solvents at 30°C. Points are means ($n = 2$).

of BP is comparable with the literature with maximum stability around pH 6.5[21].

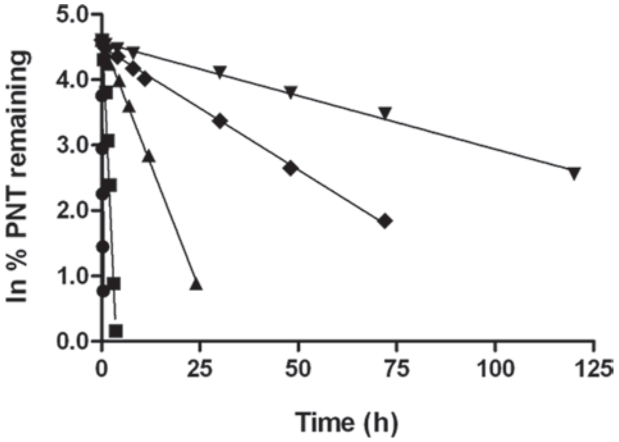


Figure 2. Observed pseudo-first-order degradation kinetics of penethamate (PNT) in various buffer solutions (50 mM, μ = 0.15; propylene glycol [PG] = 5%) of different pH at 30 ± 0.1°C. (▼) pH 4.52 (◆) pH 5.51 (▲) pH 6.02 (■) pH 7.03 (●) pH 9.31. Points are means ($n = 2$) and the lines are best-fit regression lines.

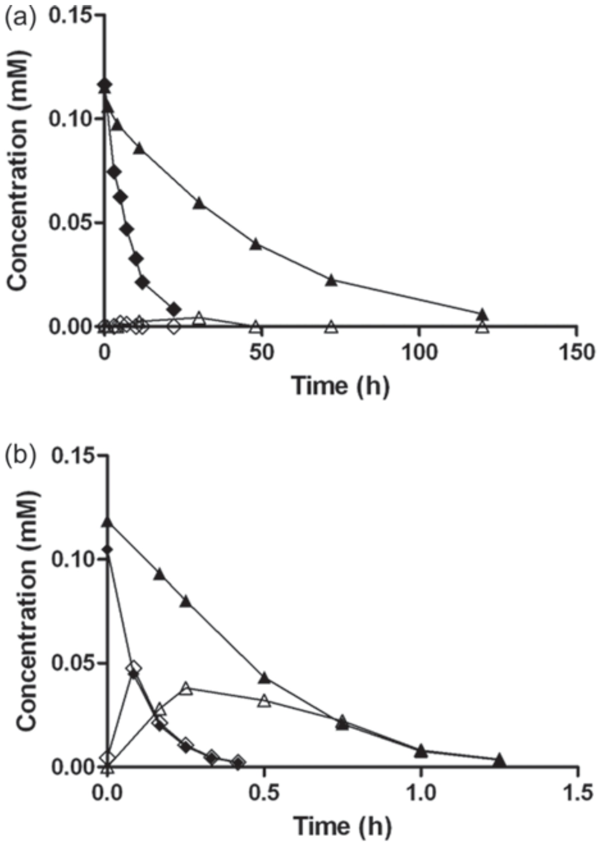


Figure 3. (A) Concentration versus time profiles of penethamate (PNT) and its degradation product benzylpenicillin (BP) in buffer solutions (50 mM, μ= 0.15; propylene glycol [PG] = 5%) at 30 ± 0.1°C. Keys: PNT at (◆) pH 3.01, (▲) pH 4.02. BP at (◇) pH 3.01 and (△) pH 4.02. Points are means ($n = 2$). (B) Concentration versus time profiles of PNT and its degradation product BP in buffer solutions (50 mM, μ= 0.15; PG = 5%) at 30 ± 0.1°C. Keys: PNT at (▲) pH 7.52, (◆) pH 9.01 and BP at (△) pH 7.52, (◇) pH 9.01. Points are means ($n = 2$).

pH-rate profile

The overall fitted pH-rate profile (not corrected for buffer catalytic effects) generated from the sum of the rate constants of PNT ($k_{PNTBP} + k_{PNTDeg}$) agreed closely with the experimental data (Figure 4) with a V-shaped curve in the pH range of 2.0–7.0 and an inflection in the pH range 7.5–9.3 which was consistent with the pK_a of PNT of 8.4.

Buffer and ionic strength effects

The chemical stability of PNT was found to be subject to general acid-base catalysis by phosphate buffer (Figure 5). The rate constants ($k_{PNTBP} + k_{PNTDeg} = k_{obs}$) were determined by treating the data according to Scheme I and plotted against increasing buffer concentration. Phosphate buffers (pH 6–8) had a catalytic effect on the degradation of PNT, however, only a slight catalysis was observed in phosphate (pH 3.0), citrate (pH 4.5) and acetate buffer (pH 4.5 and 6.0) on the rate of degradation of PNT.

Table 2. Rate constants for various reactions in the degradation of PNT in buffer solutions (50 mM, μ = 0.15; PG = 5%) of different pH at 30°C. Data are best-fit estimates ± SE from the fit.

pH	k_{PNTBP} (10^{-3} h^{-1})	k_{PNTDeg} (10^{-3} h^{-1})	k_{BPDeg} (10^{-3} h^{-1})	R^2
2.03	a	868 ± 3.2	a	0.99
3.01	a	127 ± 2.5	a	0.99
4.01	a	22.8 ± 0.7	a	0.99
4.52	4.82 ± 3.4	11.9 ± 3.4	53.2 ± 4.5	0.99
5.01	12.5 ± 1.2	9.54 ± 1.3	15.8 ± 2.6	0.99
5.51	36.7 ± 2.0	5.80 ± 2.5	13.9 ± 2.1	0.98
6.02	85.4 ± 2.5	4.15 ± 3.0	11.3 ± 2.7	0.99
6.51	223 ± 10	207 ± 12	5.60 ± 3.0	0.98
7.03	693 ± 59	202 ± 74	97.7 ± 53	0.95
7.52	1173 ± 240	a	2207 ± 453	0.95
8.04	1120 ± 290	a	1833 ± 430	0.98
9.01	6328 ± 650	a	14780 ± 1600	0.91
9.31	9800 ± 850	a	30280 ± 4000	0.93

PNT, penethamate; PG, propylene glycol; a, this degradation pathway not fitted at this pH.

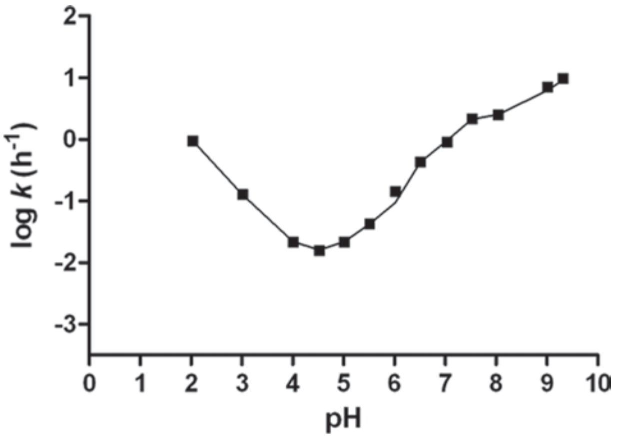


Figure 4. Overall pH-rate profile of penethamate (PNT) in various buffer solutions (0.05 M, μ = 0.15; propylene glycol [PG] = 5%) at 30 ± 0.1°C. Points are means of observed rate constant (k_{obs}; $n = 2$). The solid line is the calculated line using $k = k_{PNTBP} + k_{PNTDeg}$.

For ionic strength effects, a widely applied[19,22–24] approach as described in Equation 2 was used. Figure 6 shows the salt effect on the degradation of PNT in pH 3.01 and 9.01 solutions by plotting log k_{obs} versus $\sqrt{\mu}$ / (1 + $\sqrt{\mu}$). Increasing the ionic strength caused a small but significant increase in degradation rate at pH 3.01. The slope of the regression line was not significantly different from the theoretical slope of +1.026 expected for the hydronium ion-catalyzed degradation of the protonated cationic form of PNT. At pH 9.01, where the predominant reaction is likely to be the hydroxyl ion-catalyzed hydrolysis of the unionized form of PNT, the salt effect was negligible as expected.

Temperature effects

The temperature dependence of PNT degradation at phosphate (pH 3.01 and 8.04) and acetate (6.01) buffer solutions (50 mM, $\mu = 0.15$) was studied in the temperature range 30–50°C. Arrhenius plots were linear ($R^2 \geq 0.99$; Figure 7) with activation energies at pH 3.01, 6.01 and 8.04 of 62.1, 74.1 and 98.8 kJ mol^{-1}, respectively.

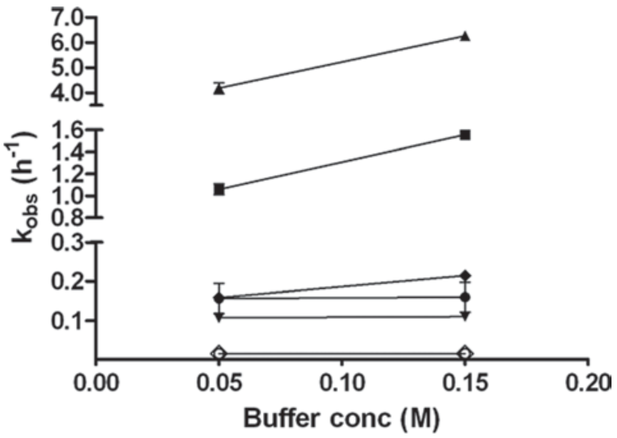


Figure 5. Effect of buffer concentration and buffer type on the pseudo-first-order rate constants of the degradation of penethamate (PNT) at fixed pH values (30 ± 0.1°C, $\mu = 0.5$). (•) Phosphate pH 3.01, (■) Phosphate pH 7.15, (▲) Phosphate pH 8.04, (◆) Phosphate pH 6.01, (▼) Acetate pH 6.01, (○) Acetate pH 4.52, (◇) Citrate pH 4.52. Points are means ($n = 2$).

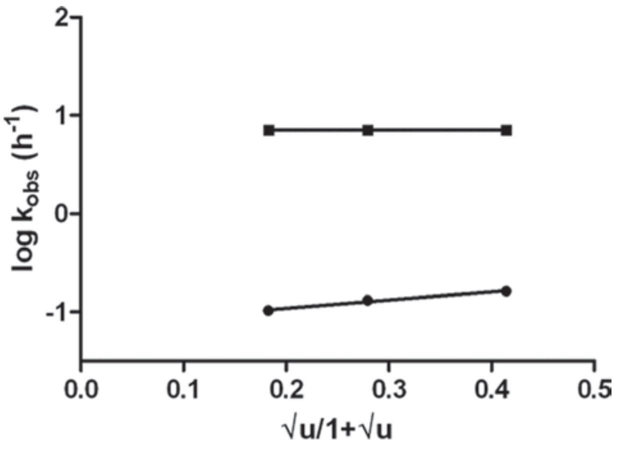


Figure 6. Salt effect on degradation of penethamate (PNT) at (●) pH 3.01 and (■) pH 9.01 at 30 ± 0.1°C. Points are means ($n = 2$).

Solvent effects and stability in solution

The chemical stability of PNT in different cosolvent systems followed pseudo-first-order kinetics. The stability of PNT in solution in the cosolvent systems is shown in Table 3. The degradation rate constant of PNT in the PG-citrate buffer system decreased as the content of PG increased (Table 3), with the half-life ($t_{1/2}$) for PNT at pH 4.5 in 60:40 PG:citrate buffer cosolvent system being 105 h (4.4 days) compared with 43 h (1.8 days) in pH 4.5 buffer alone.

Discussion

The results allow assessment of the potential of developing a novel ready-to-use aqueous-based PNT formulation in future. The degradation of PNT in aqueous solutions followed pseudo-first-order kinetics over the pH range 2–9.3 and a V- shaped pH-rate profile was observed. Similar profiles for ester prodrugs are reported in the literature[25,26]. The impact of the investigated buffer concentration and ionic strength on PNT stability was small, but at pH 6, the type of buffer salt had some influence with solutions in acetate being about twice as stable as those in phosphate at the higher buffer concentration (Figure 5). However, the major effects on stability were pH, cosolvent (PG) and temperature, so the following discussion focuses on these factors. Initially, the PNT degradation kinetics will be discussed to emphasize the importance of using the recently developed stability-indicating HPLC assay for PNT[10] for investigating the chemical stability of the prodrug itself.

In the acidic region (pH 2–4), degradation of PNT was not accompanied by an equivalent detectable formation of BP; in fact, BP concentrations were very low (Figure 3A). This indicates that PNT may be hydrolysing to products other than BP (e.g. cleavage of the β-lactam ring of PNT) or may be hydrolysing to BP which then

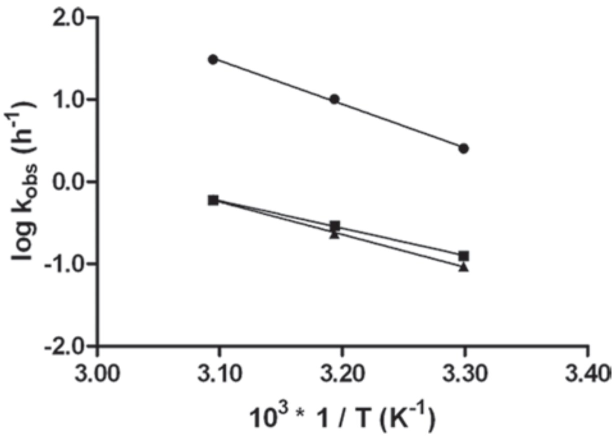


Figure 7. Arrhenius plots showing effect of temperature on the reaction rates of hydrolysis of penethamate (PNT) at various pH. (▲) pH 3.01, (■) pH 6.01, (•) pH 8.04. Points are means ($n = 2$).

rapidly sequentially hydrolyses, or degradation may be a combination of these pathways. The literature reports degradation rates of BP at pH 4 for diverse temperatures[27] from which a degradation rate of BP of 143×10^{-3} h^{-1} for 30°C was calculated. Taking this rate constant into consideration and assuming that PNT only degrades to BP, calculations suggests that BP concentrations should have been significantly higher than those observed in this study. This suggests that at low pH (pH 2–4), PNT degradation to BP is minimal and it mainly degrades by alternative pathways.

In the intermediate region (pH 4.5–7.0), degradation of PNT involved all the pathways according to Scheme I so that PNT degraded to BP and other degradation products. The BP so formed was estimated to have a half-life of 6.2 days at pH 6.5, which is somewhat shorter than the 38 days reported for BP at pH 6.75 at 30°C[21]. However, the present study was aimed at assessing the stability of PNT, not BP, and therefore experimental conditions were not optimized for BP stability analysis.

In the alkaline region (pH 7.5–9.3), a high concentration of BP was detected, indicating that the major reaction involves cleavage of the ester group due to hydroxide ion attack (Figure 3B). BP also subsequently degraded. The reported degradation rates (k_{obs}) of BP at pH 9.0 of 8.6×10^{-3} h^{-1} (25°C) and 30×10^{-3} h^{-1} (35°C), respectively[27], are somewhat slower than those estimated here at 30°C. The inflection point in this pH region is at the pK_a of PNT (8.4). Inflections are frequently observed near the pK_a of substrate, when the reactivity of protonated and non-protonated forms are sufficiently different. Similar profiles have been reported for β-lactam antibiotics and reflect changes in the mechanisms of degradation with changes in pH[28–30].

Given that PNT degrades not only to BP (Scheme 1), and given that BP itself degrades, any assessment of PNT stability which is based solely on analysis of BP must be misleading. The stability assessments of the early literature were based on spectrophotometric or biological assays of BP and therefore underestimated the degradation of PNT. Dinsmore and Bailey[11] used change in partition coefficient or change in solubility together with a spectrophotometric assay to estimate stability at 25°C. At pH 3–4 (100 mM buffer), they reported about 10% degradation over a period of 2–2.5 h; however, the present study indicates 18% degradation of PNT over a period of 2–3 h and degradation of BP. Hallas-Moller et al.[12] reported 39% degradation of PNT in 22 h at pH 5 and 37°C, whereas the present study estimates 68% degradation in this time. Consequently, the development of a ready-to-use aqueous-based PNT formulation must be assessed on the prodrug itself.

The addition of PG improved the stability of PNT, possibly because decreasing the polarity of the reaction medium favors uncharged species and thereby stabilizes a solute against any reaction that produces charged products or proceeds through a charged transition state[31].

The above discussion of the chemical stability of PNT was focused only on PNT solutions. To further assess the potential development of a ready-to-use aqueous-based PNT suspension formulation or extend the storage life of the reconstituted injection, shelf lives (t_{90}) of PNT suspensions were estimated as follows:

$$t_{90} = 0.1 \times \frac{C_o}{k_o} \tag{6}$$

where C_o is the initial PNT concentration in the suspension and k_o is the apparent zero-order rate constant. The zero-order rate constant is calculated as follows[32]:

$$k_o = k_{obs}[PNT_{sol}] \tag{7}$$

where k_{obs}, the apparent first-order rate constant, is dependent on the PG level (Table 3), and $[PNT_{sol}]$ is the solubility of PNT in the relevant mixed solvent system (Table 1). So shelf life can be increased by using a high-concentration suspension (i.e. high C_o) and by minimizing PNT solubility and degradation rate in solution. C_o is limited by practical issues such as the injectability of the product. Degradation rate in solution can be minimized by judicious choice of pH, buffer type, storage at low temperature and by addition of PG. However, addition of PG also increases solubility thereby compromising its benefit to increase stability in solution.

The shelf life was estimated for aqueous-based PNT suspensions containing up to 50% drug content and up to 60% cosolvent content for a temperature range of 5°C to 30°C and pH 4.5. The required first-order rate constants, k_{obs}, for pH 4.5 at temperatures 5°C and 20°C were estimated by applying the following procedure and assumptions:

- A polynomial was fitted to the V-shape pH-rate profile for 30°C.
- The same polynomial, except for the intercept term, was fitted to the limited pH-rate data (pH 3.01, 6.01 and 8.04) for 40°C and for 50°C. The change in the intercept corresponding to a vertical dislocation seems

Table 3. Stability of PNT in PG:citrate buffer (50 mM) solvent systems at different pH and 30°C.

PG:buffer (v/v)	Mole fraction of PG	pH 3.01		pH 4.52		pH 6.01	
		k_{obs} (10^{-3} h^{-1})	$t_{1/2}$ (h)	k_{obs} (10^{-3} h^{-1})	$t_{1/2}$ (h)	k_{obs} (10^{-3} h^{-1})	$t_{1/2}$ (h)
20:80	0.057	170	4.0	10.2	68.0	50.7	13.6
40:60	0.137	110	6.3	9.40	74.0	45.8	15.1
60:40	0.265	80.7	8.6	6.60	105	43.1	16.0

PNT, penethamate; PG, propylene glycol, k_{obs}, observed rate constant.

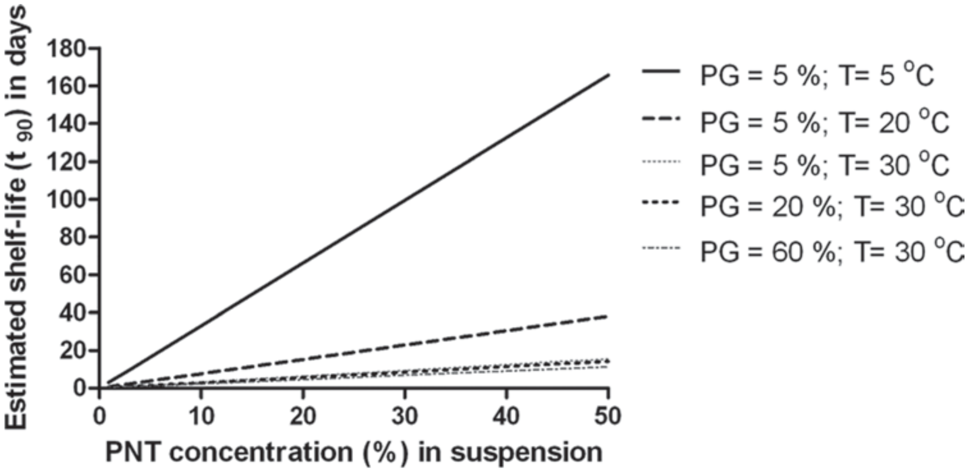


Figure 8. Estimated shelf life for penethamate (PNT) suspensions with different % propylene glycol (PG) and temperatures at pH 4.5.

an acceptable assumption as Connors et al.[21,33] have reported a similar approach of retaining the shape of pH-rate profiles at different temperatures with a dislocation in vertical direction for other drugs, e.g. BP and indomethacin[21,33]. Consequently, rate constants k_{obs} for 40°C and 50°C at pH 4.5 were determined.

- Finally, rate constants for 5°C and 20°C were extrapolated from Arrhenius plot for pH 4.5.

Figure 8 shows the shelf life (t_{90}) over PNT concentration in suspensions for pH 4.5 depending on cosolvent PG content and temperature. With increasing PNT concentration in PG colsovent mixtures, the shelf life increases linearly from about 0.3 days (5% PG) and 0.7 days (60% PG) in solution to about 14 days (5% PG) and 11 days (60% PG) in a 50% PNT suspension. Although PG improves the stability of PNT in solution, its addition to a suspension formulation does not result in enhanced stability, at least at pH 4.5, of the formulation as PG increases the solubility of PNT. The overall impact of PG cosolvent content at 30°C on the stability of PNT suspension is insufficient for product stability if ICH guidelines[34] would be applied for accelerated conditions (30°C, 65% RH). On the other hand, the shelf life increases with decreasing temperature and increasing PNT concentration in suspension (5% PG). A best case scenario was calculated for a 50% PNT suspension in acetate/citrate buffer (pH 4.5, 50 mM) containing 5% PG for a temperature of 5°C, suggesting a storage life (t_{90}) of about 164 days or 5.5 months could be achieved.

Conclusions

Current PNT formulations must be reconstituted before use and the reconstituted injection has a short storage life. A recently developed HPLC assay for PNT in aqueous solutions was used to study the degradation kinetics of PNT under various formulation and storage conditions. The results emphasize the need to analyze the prodrug PNT itself, and not base assessments on the drug BP. PNT degradation pathways depend on the pH. The major formulation/storage factors influencing the chemical stability of PNT in solution are pH, PG content and storage temperature. Buffer type, buffer concentration and ionic strength have less influence on stability. Judicious choice of the suspension concentration and the PG content suggests that a shelf life of 5.5 months at 5°C could be achieved.

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Declaration of interest

The authors report no declarations of interest.

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