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A stability-indicating HPLC assay with diode array detection for the determination of a benzylpenicillin prodrug in aqueous solutions

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ABSTRACT

The aim of this study was to develop a stability-indicating HPLC assay for the determination of penethamate (PNT), an ester prodrug of benzylpenicillin (BP), in aqueous solutions. The method was validated by subjecting PNT to forced decomposition under stress conditions of acid, alkali, water hydrolysis and oxidation. A quenching solution was developed to limit degradation to negligible levels before and during the analysis. Both PNT and BP were simultaneously determined and separated in presence of degradation products on a C_{18} column using a mobile phase consisting of methanol–acetonitrile–acetate buffer. Different degradation products were formed in the stress conditions. The peak purity indexes of PNT and BP obtained by diode array detection were >0.999, confirming the absence of other co-eluting substances. The assay was linear for both analytes in the concentration range 1–100 $\mu g\,mL^{-1}$. The LOD and LOQ of PNT were 0.03 and 0.09 $\mu g\,mL^{-1}$ respectively. Degradation of PNT followed pseudo-first-order kinetics with $t_{1/2}$ of 43.6 min at pH 2.01 and 4.2 min at pH 9.31. In addition, the absence of BP in the acidic solutions of PNT emphasises the futility of monitoring BP to assess the stability of PNT. In conclusion, the assay is rapid and stability-indicating with adequate precision and accuracy, and in conjunction with the quenching solution, can be used for stability studies of PNT with simultaneous quantitation of BP. The degradation studies provide useful information for formulation development of PNT.

1. Introduction

Penethamate (PNT) is a diethylaminoethyl ester prodrug of benzylpenicillin (BP) (Fig. 1). It is used for the treatment of bovine mastitis in intramammary products as oily suspension [1] and as an intramuscular (i.m.) injection in the form of a dry powder for reconstitution prior to use [2]. The antimicrobial activity of PNT is due to BP with a spectrum of activity primarily against non-penicillinase producing gram-positive cocci mainly *Streptococcus uberis*, *Streptococcus dysgalactae*, *Streptococcus agalactae* and penicillin sensitive *Staphylococcus aureus* [3,4]. However, the weakly basic (pK_a 8.5) [5] and lipophilic nature of PNT renders it advantages over BP in treatment of bovine mastitis [6] because of PNT's ability to diffuse through the parenchyma of the udder [7] and penetrate the mammary epithelial cells [8]. Following i.m. administration, PNT circulates in the blood with 10% in the non-ionised form. The non-ionised PNT easily passes the milk–blood barrier [5,9] but then is trapped in the milk (pH 6.4–6.6) where the majority of PNT is ionised. In addition, PNT is hydrolysed to the weak acid BP (pK_a 2.8) which is also ionised in the milk thereby limiting its return to the circulation and resulting in high concentrations in the udder [10]. Friton et al. [11] reported that the area under the concentration–time curve of BP was >2 times higher in milk than in plasma in dairy cows following i.m. injection of Mamyzin®, the commercial formulation of PNT.

PNT, like other β-lactam antibiotics, has a fused β-lactam-thiazolidine ring system susceptible to degradation involving various nucleophiles, acid–base reagents, metal ions, oxidizing agents and solvents [12,13]. In addition, the presence of the ester prodrug group adds to its hydrolytic instability with formation of BP and diethylaminoethanol (Fig. 1). Jensen et al. [14] reported that the half life ($t_{1/2}$) of PNT was 23 and 220 min at pH 7.3 and pH 6.2 respectively in a 0.9% NaCl solution at 37 °C. Dinsmore and Bailey [15] determined the rates of hydrolysis of PNT in solutions at pH 2–7.5 and reported that PNT is least hydrolysed at pH 4.9 with <10% hydrolysed after 3 h at 25 °C. Improved stability of PNT was reported in a suspension (pH 6) with 7% degraded after 7 days at 25 °C [16]. However, these reports on the stability [14–16] or pharmacokinetics [11,17,18] of PNT were based on the analysis of BP, rather than PNT itself by UV spectrometry [14–17] and a liquid chromatographic mass spectrometry (LC–MS) assay [18]. The current EMEA guidance prefers analyses of parent drug as well as the active metabolite

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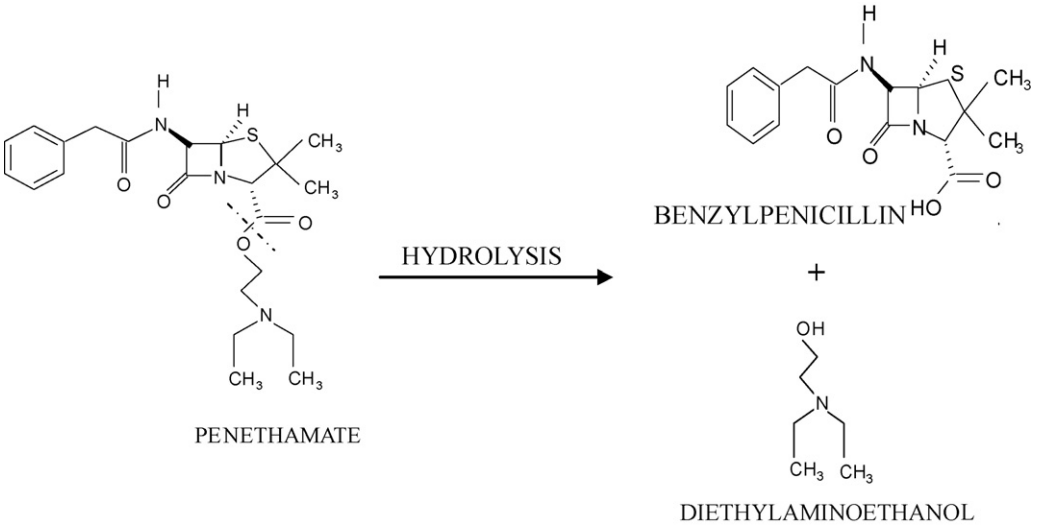

Fig. 1. Schematic of the hydrolysis of PNT to give BP and diethylaminoethanol.

[19] but, there is no stability-indicating HPLC assay specific for the determination of PNT reported in the literature.

On the other hand, there are many studies on the stability of BP in acidic, alkaline and neutral conditions [12,13,20–25]. Also many HPLC and LC–MS methods for the detection of BP in the presence of its degradation products or other β-lactam antibiotics [26–32] have been published.

The stability of PNT as a function of pH is an important consideration in the formulation development particularly with regard to the formation of BP. The drug stability test guideline Q1A (R2) issued by the International Conference on Harmonization (ICH) [33] requires that analytical test procedures should be stability-indicating and that they should be fully validated. The aim of the present study was to determine the stability of PNT under a variety of ICH-recommended stress-testing conditions and to develop a stability-indicating assay [34] that is capable of separating PNT and BP from the degradation products. Further the application of this method is shown by its ability to assess degradation kinetics of PNT in aqueous solutions.

2. Experimental

2.1. 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, USA. 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, USA) was used for mobile phase and stability sample preparations. All other chemicals and solvents were of analytical grade (BDH Chemicals Ltd., England, or Ajax Finechem, New Zealand).

2.2. Instrumentation and chromatographic conditions

The HPLC instrument employed was a Shimadzu LC-20 Prominence series (Shimadzu, Kyoto, Japan) LC system equipped with a LC-20AD quaternary pump, a DGU-20A5 degasser unit, a SPD-M20A diode array detector, a SIL-20AC auto-injector (set at 4 °C) and a CTO-20A column oven (set at 40 °C). The output signal was monitored and processed using Class VP 7.4 software (Shimadzu, Kyoto, Japan). The injection volume was 100 μL and chromatographic separation was achieved on a Luna $C_{18,}$ 150 mm × 4.6 mm i.d., 5 μm particle size column (Phenomenex, New Zealand), using methanol acetonitrile–acetate buffer (pH 5; 50 mM) (35:5:60, v/v/v) as mobile phase at a flow rate of 1 mL min^{-1}. PNT and BP were determined by UV detection at 230 nm.

2.3. Development of quenching solution

For method validation, it was necessary to develop a diluent (quenching solution) to limit the degradation in samples to negligible levels before and during the analysis. In a preliminary study and later confirmed by the validated reversed phase HPLC method, PNT was found to degrade most rapidly in borate buffer (pH 10; 50 mM) (20% remained after 1 h at 0 °C) and most stable in acetate buffer at pH 4.8 (88% remained after 24 h at 0 °C) compared to solutions prepared in different buffers (phosphate, acetate) at pH 2 to 10. Combinations of solvents (50–80%, v/v PG or ethanol) with acetate buffers (50 mM to 1 M) were investigated as an appropriate diluent of PNT samples at temperatures −80, 0, 4 and 25 °C in an effort to develop a quenching solution in which PNT was stable for at least 24 h. Finally, a quenching solution containing PG–acetate buffer (1 M) (50:50, v/v) (pH 4.8) was selected for method validation. At a ratio of 9:1 (v/v) to sample solution, the quenching solution was able to stabilise a PNT solution in borate buffer (pH 10; 50 mM). That is, 98 ± 1% PNT remained after 24 h at 0 °C and 99 ± 1% after 3 months at −80 °C. Similarly 98.2 ± 1% of BP was detected after 24 h in the quenching solution at 0 °C.

For the preparation of stock solutions, the stability of PNT was determined in various cosolvent systems (water, PG, PEG 400 or ethanol or their combinations) at different temperatures. The stability of PNT was in order PG > PEG > ethanol > water. Therefore, PG was selected in which 98.5 ± 1% PNT remained after 24 h at 0 °C and 48 h at −20 °C.

2.4. Stress degradation of PNT

Stress degradation studies of PNT were performed in accordance with International Conference on Harmonisation (ICH) guidelines [33] in order to demonstrate the stability-indicating [34] feature of the assay. The degradation products were induced in acid, base, water and oxidation conditions. The experiments were carried out as follows: to 4 mL of a 1 mg mL^{-1} solution of PNT in PG, 21 mL of HCl or NaOH or water or hydrogen peroxide (H_2O_2) solutions of different concentrations were added. These samples, prepared in

duplicate, were protected from light and stored at 25 °C. pH was measured at the beginning and end of the experiment and no pH change in the samples was observed except in water where pH was changed from 6.98 to 6.28. Aliquots were taken at appropriate time intervals depending on decomposition rate (until 0–60% of PNT remained), diluted with the quenching solution and analysed immediately for the determination of PNT and degradation products.

2.5. Stock solutions of PNT and BP

For method validation a stock solution of PNT (1 mg mL^{-1}) was prepared in the quenching solution. Working solutions of 200 μg mL^{-1} were prepared from the stock solution for the assay. A 1 mg mL^{-1} stock solution of BP was also prepared in quenching solution. Stock solutions were stored at −80 °C.

2.6. Validation of method

Method validation was carried out in accordance to ICH [35] and Australian Pesticides & Veterinary Medicines Authority (APVMA) guidelines [36].

2.6.1. Specificity

The specificity of the assay was determined by the complete separation of PNT in the presence of its degradation products generated under stress conditions. The asymmetry factors of the peaks were calculated from freshly prepared samples of PNT and BP. The peak purity test was carried out on stressed samples using the diode array detector. The PNT chromatographic peaks were corrected for background, and the wavelength range 215–340 nm was used to calculate peak–purity and similarity index.

2.6.2. Linearity

The assay was validated over the PNT concentration range of 1–100 μg mL^{-1}. Triplicate stock solutions of PNT were prepared in the quenching solution (200 μg mL^{-1}) and diluted with quenching solution to give analytical standards at six concentration levels (1, 5, 10, 25, 50 and 100 μg mL^{-1}). Standard curves were constructed using standard regression techniques. All the standard solutions were stored in slushy-ice bath (0 °C) protected from light and analysed within 4 h of preparation.

2.6.3. Precision

The intra-day and inter-day variabilities were determined by repeated injections of quality control (QC) standards of PNT at concentrations of 3, 20 and 80 μg mL^{-1} in quenching solution prepared from a different stock following the same procedure as for analytical standards. QC standards were protected from light at −80 °C (until analysed), thawed at 0 °C and then analysed. This was repeated on 5 consecutive days.

2.6.4. Accuracy

Accuracy was accessed by comparing the predicted concentrations of the QC standards, using the standard curve, with the theoretical concentrations (3, 20 and 80 μg mL^{-1}).

2.6.5. Limit of detection (LOD) and limit of quantitation (LOQ)

LOD and LOQ were determined on the basis of standard deviation (σ) of the response and slope (S) of the calibration curve using 3.3 σ/S and 10σ/S respectively.

2.6.6. Linearity, LOD and LOQ for BP

The linearity for BP was investigated in the concentration range 1–100 μg mL^{-1} in triplicate following a similar procedure to that used for PNT. LOD and LOQ were determined on the basis of standard deviation (σ) of the response and slope (S) of the calibration curve using 3.3σ/S and 10σ/S respectively.

Table 1
Effect of stress conditions on degradation of PNT at 25 °C.

Stress condition		Time (h)	PNT remaining (%)
Acidic	1 mM HCl	22	53.6
	10 mM HCl	22	0.04
Water		480	14.2
Alkaline	0.1 mM NaOH	22	59.0
	1 mM NaOH	22	44.3
	10 mM NaOH	2	2.0
Oxidation	0.3%, v/v H_2O_2	2	24.2
	3%, v/v H_2O_2	1	10.5

2.7. Degradation kinetics studies

To show its application, the validated HPLC method was utilised in the degradation kinetics studies of PNT. Phosphate (pH 2.01) and borate (pH 9.31) buffers (50 mM) were adjusted to constant ionic strength (0.15) with sodium chloride. The kinetic studies were carried out as follows: 9.5 mL of the buffer in a 25 mL stoppered Erlenmeyer flask was placed in water-bath and pre-warmed at 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 reaction mixture were taken and added to 4.5 mL of quenching solution in a 0 °C slushy-ice bath to quench the reaction. The samples were analysed immediately using the stability-indicating HPLC method for simultaneous determination and quantitation of PNT and BP. Duplicate samples were used for each storage condition in this study.

3. Results

3.1. Development and optimisation of stability-indicating method

For mobile phase development, initially only methanol and buffer in different ratios and pH were tried. A mobile phase consisting of methanol–acetate buffer (pH 5; 50 mM) (40:60, v/v) gave symmetrical peaks and satisfactory resolution between PNT and BP. Acetate buffer (pH 5; 50 mM) was selected as both PNT (pK_a 8.5) and BP (pK_a 2.8) are ionised at this pH and the buffer provided good buffer capacity resulting in stable retention times (t_R). However, the resolution between BP and degradation products was poor. The method was optimised by addition of 5% acetonitrile to the mobile phase, which resulted in shorter retention times and improved resolution with degradation products for both PNT and BP. Ultimately a mobile phase consisting of methanol–acetonitrile–acetate buffer (pH 5; 50 mM) (35:5:60, v/v/v) was selected. This mobile phase gave a good resolution between PNT (t_R = 10.3 min) and its major hydrolysis product BP (t_R = 7.3) with no interference from other degradation products formed in the stress conditions.

3.2. Stress degradation of PNT

Under stress conditions the PNT peak reduced over time (Table 1) with appearance of different unknown degradation product peaks (I–IX) (Fig. 2).

3.2.1. Acidic conditions

PNT degraded in acidic conditions (Table 1) with appearance of degradation products II, IV, V, and IX (Fig. 2a). The absence of BP in the samples suggests that either any BP formed degraded

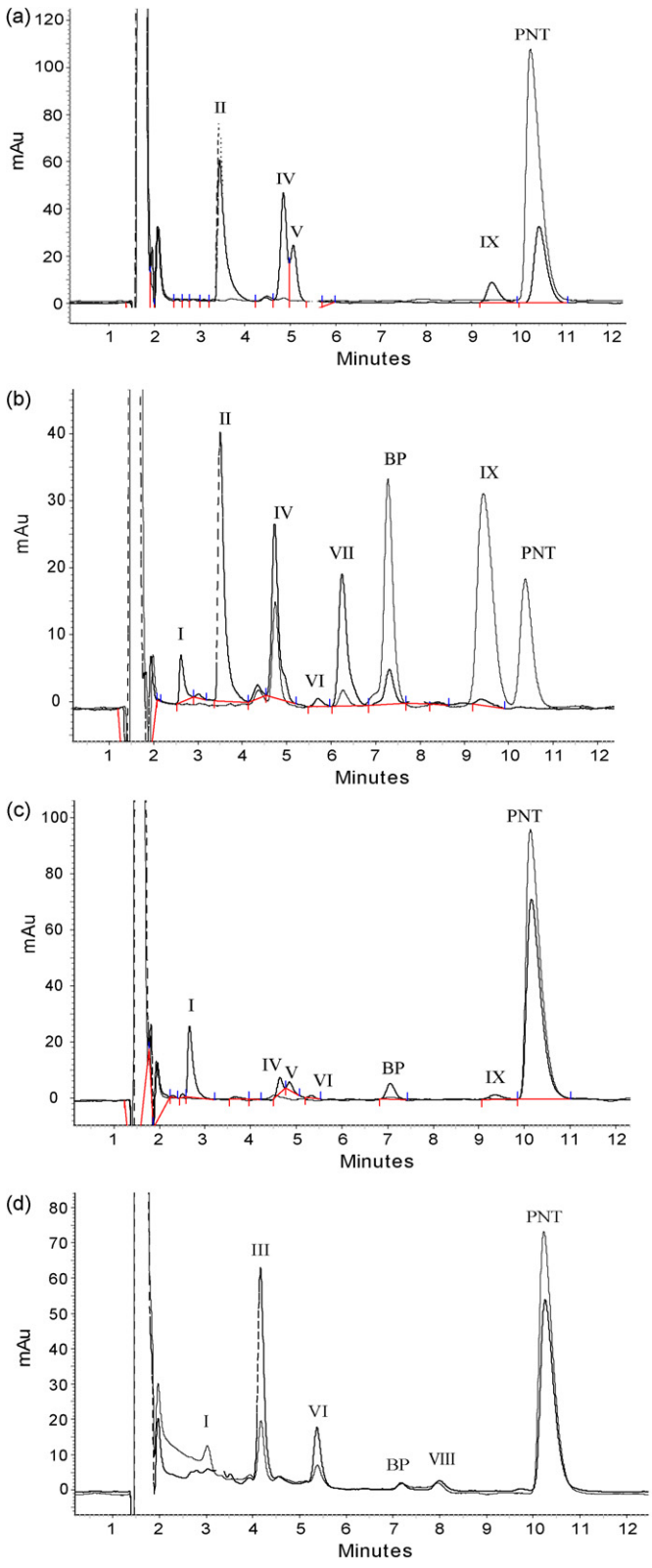

Fig. 2. Overlay chromatograms showing degradation of PNT and in some cases formation and sequential degradation of BP and unknown degradation products at various times (solid lines represent samples at time 0) under different stress conditions: (a) 10 mM HCl, (b) 1 mM NaOH, (c) water, and (d) 0.3% (v/v) H_2O_2 solution.

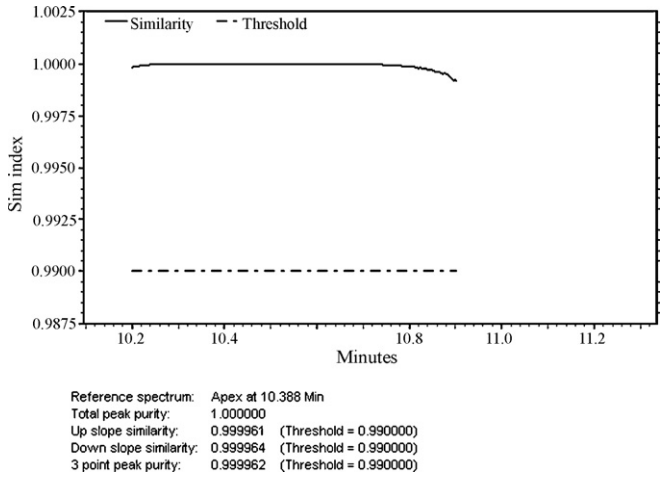

Fig. 3. Typical diode array peak purity profile showing peak purity and similarity index for PNT.

rapidly or the hydrolysis of PNT to BP is negligible and degradation of PNT occurred predominantly by alternative pathways in the acidic solutions.

3.2.2. Alkaline conditions

PNT was found to degrade more rapidly in the alkaline solutions than in the acidic solutions (Table 1). In 1 mM NaOH solutions degradation occurred even during sample preparation with rapid appearance of peaks I, II, III, IV, VI, VII, BP and IX (Fig. 2b). A significant amount of BP was observed indicating hydrolysis of the ester occurred under alkaline conditions.

3.2.3. Water conditions

In water, degradation occurred at a slower rate compared to acidic and alkaline conditions with appearance of I, IV, V, BP and IX. However, in the later stages another peak (VI) emerged which was also present in basic conditions but absent in acidic conditions (Fig. 2c).

3.2.4. Oxidative conditions

PNT degraded most rapidly in the presence of H_2O_2 (Table 1) with a rapid formation of degradation peak III. In addition, a new degradation peak VIII was also seen in the chromatograms (Fig. 2d).

3.3. Validation of stability-indicating HPLC method of PNT

3.3.1. Specificity

The HPLC method was found to be specific with complete separation of PNT (t_R 10.3 ± 0.1 min) from its degradation products under all degradation conditions. The asymmetry factor was 1.1 and 1.0 for PNT and BP respectively. The peaks obtained were sharp with clear baseline separation. The peak purity and similarity indexes of PNT (Fig. 3) and BP under all degradation conditions were >0.999, confirming the absence of other substances co-eluting with PNT and BP. The assay of PNT was unaffected by the presence of BP and unidentified degradation products which confirmed the stability-indicating feature of the method.

3.3.2. Linearity, precision, accuracy, LOD and LOQ for PNT

The assay for PNT was found to be linear in the range 1–100 μg mL^{-1} with a high correlation coefficient ($r > 0.999$). Intra-day and inter-day variabilities were low and accuracy was high (Table 2). The LOD and LOQ of PNT were 0.03 and 0.09 μg mL^{-1} respectively.

Table 2
Intra-day, inter-day precision and accuracy (%) of QC standards at 3, 20 and 80 μg mL^{-1} (PNT).

Theoretical conc. (μg mL^{-1})	Intra-day (n = 3)			Inter-day (n = 5)		
	Mean conc. ± SD (μg mL^{-1})	RSD (%)	Accuracy (%)	Mean conc. ± SD (μg mL^{-1})	RSD (%)	Accuracy (%)
3	2.98 ± 0.02	0.70	99.4 ± 0.65	2.98 ± 0.02	0.88	99.2 ± 0.69
20	19.76 ± 0.09	0.43	98.8 ± 0.43	19.76 ± 0.19	1.00	99.0 ± 0.83
80	78.74 ± 0.33	0.42	98.4 ± 0.42	78.46 ± 0.17	0.22	98.2 ± 0.85

RSD (%) = SD/mean × 100.

3.3.3. Linearity, LOD and LOQ for BP

The assay for BP was found to be linear in the range 1–100 μg mL^{-1} with a high correlation coefficient (r > 0.999). The LOD and LOQ of BP were 0.14 and 0.42 μg mL^{-1} respectively.

3.4. Degradation kinetic studies

Stability profiles for PNT and its hydrolysis product BP in different pH buffer solutions at 30 ± 0.1 °C are shown in Fig. 4. The linear relationship between logarithms of percent PNT remaining *versus* storage time indicates pseudo-first-order degradation kinetics with $t_{1/2}$ of 43.6 min at pH 2.01 and 4.2 min at pH 9.31 respectively. At pH 2.01 no BP was formed; on the other hand, at pH 9.3 a high concentration of BP, approximately 1/3 of the initial concentration of PNT, was detected at 5 min. However, BP also subsequently degraded rapidly.

4. Discussion

PNT is widely used in the treatment of bovine mastitis with favourable pharmacokinetics over BP. Although the hydriodide is soluble in aqueous solution, the high instability prevents its injectable formulation in an aqueous vehicle. At present, it is available as dry powder for reconstitution in water prior to use. After reconstitution, the shelf-life of the suspension is specified as 7 days at 2–8 °C and 2 days at 15–20 °C [2]. These shelf-lives were determined by the solubility of the PNT in the vehicle and the degradation rate of the PNT in solution. In an effort to develop a more stable formulation it is important to develop a stability-indicating HPLC method to understand the stability behaviour of PNT in aqueous solutions. Also, there are no pharmacokinetic studies [11,17,18] in which the PNT parent compound has been analysed.

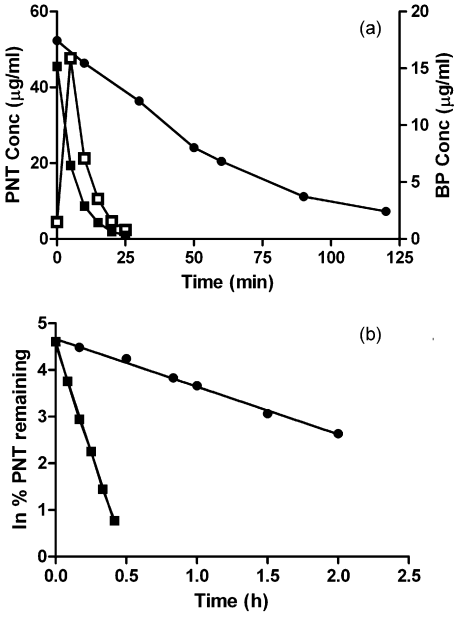

Fig. 4. Concentration *versus* time profiles (a) and first-order plots (b) of PNT and its degradation product BP in buffer solutions (0.05 M, μ = 0.15) at 30 ± 0.1 °C. Keys: (●) pH 2.01, (■) pH 9.31, and (□) BP at pH 9.31 (BP was not detected at pH 2.01). Points are mean (n = 2).

Due to its extreme instability in aqueous solutions particularly at high pH, there was a need to prepare a quenching solution in which degradation of PNT was limited to a negligible level so that the HPLC assay and validation could be performed. A quenching solution was also required for the degradation studies. In this study, PNT was found to be relatively stable in pure PG; however, with addition of an alkaline solution of PNT to PG, the drug continued to degrade at an unacceptable rate with 50% remained after 24 h at 0 °C. On the contrary, presence of 50% 1 M acetate buffer (pH 4.8) in PG conferred satisfactory stability to PNT at low temperatures. This indicates that the pH and buffer capacity are necessary to the quenching solution. These results also provide useful information for formulation development.

In the stress degradation studies, overlaying of chromatograms (Fig. 2) clearly explains the disappearance of PNT and formation of degradation products under various conditions; acidic, water, alkaline hydrolysis and oxidation, suggesting that the assay is stability-indicating and capable of separating PNT in the presence of potential degradation products. The peak purity and of PNT and BP under all these degradation conditions was greater than 0.999, confirming the absence of other co-eluting substances. The assay is linear in the concentration range 1–100 μg mL^{-1} (r > 0.999). Both intra-day variability (n = 3) and inter-day variability (n = 5) at 3.0, 20.0 and 80.0 μg mL^{-1} were satisfactory (RSD ≤1%). The mean percentage accuracy at above concentration levels was 98–99%.

The degradation pathways of BP are complex as elucidated in earlier studies [12,13,20–25]. In the current study, some efforts have been made to correlate the results with these studies. PNT, being an ester prodrug of BP, may follow similar degradation pathways to BP after hydrolysis to BP; however, there are limited data in the literature that discuss the fate of PNT in different pH conditions and the assays were based on spectrophotometric determination of BP [14–16].

In the acidic solutions of PNT, no BP was observed. Instead, four other degradation products were seen. This may be due to the high instability of BP in acidic solutions [12,13,21–25] or the ester hydrolysis of PNT to BP is negligible and PNT degraded predominantly by alternative pathways. In either case, this result emphasises the futility of monitoring BP to assess the stability of PNT, and the need for a stability-indicating assay for PNT. Formation of BP in water and alkaline solutions is attributed to the hydrolysis of the ester group of PNT. The BP so formed may have resulted in formation of penicilloic and penicillenic acid, which are known to be formed in alkaline conditions [12,13,20,22–24] and again emphasizing the need for a stability-indicating assay for PNT. Similar conclusions apply to oxidative conditions. In the presence of H_2O_2, degradation peaks III and VIII were observed. These degradation products may be attributed to the oxidation of the sulphur and/or the tertiary amine present in the structures of PNT and BP.

Low (pH 2.01) and high (pH 9.31) pH were selected for degradation kinetics studies as the degradation of PNT is maximum at these pH and indicate the applicability of this method for carrying

out further degradation studies at other pHs (pH 2–10). At high and low pH, degradation of PNT followed pseudo-first-order kinetics and degradation of PNT occurred more rapidly at alkaline pH than at acidic pH. The chromatograms confirmed that little or no BP was formed at pH 2.01. In contrast, at pH 9.31 a high ratio of PNT was converted into BP, however, it also subsequently degraded rapidly.

The stability-indicating assay developed for PNT is an important addition in the scarce scientific literature on PNT, since this prodrug is a useful molecule and yet there is no stability-indicating assay for it in the literature. The method is particularly valuable due to its ability of separating PNT and BP simultaneously in the presence of the other degradation products, and hence in combination with the quenching solution enabled quantification of PNT and BP in the degradation samples.

5. Conclusions

The stability-indicating reversed phase HPLC assay for PNT is rapid, reliable and capable of quantifying both PNT and BP in presence of other potential degradation products of PNT formed in acidic, water, alkaline and oxidation conditions. The assay is linear in the concentration range 1–100 $\mu g\,mL^{-1}$ with adequate precision and accuracy. The degradation of PNT followed pseudo-first-order kinetics with $t_{1/2}$ of 43.6 min at pH 2.01 and 4.2 min at pH 9.31 respectively. In the acidic solutions, no BP was observed in the degradation samples of PNT. This suggests that the other methods that monitor only BP are not suitable to assess the stability of PNT. In summary, the HPLC assay is simple, stability-indicating and in conjunction with quenching solution, can be used for stability studies (pH 2–10) and routine analysis of PNT and for simultaneous quantitation of BP in aqueous solutions. The degradation studies provide useful insight for formulation development of this prodrug.

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