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A rapid and sensitive method for determination of dimethyl benzoylphenyl urea in human plasma by using LC/MS/MS

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Abstract

Dimethyl benzoylphenyl urea (BPU), a poorly water-soluble benzoylphenyl urea derivative, inhibits tubulin polymerization and causes microtubule depolymerization in vitro with activity against solid tumors. BPU is currently being tested in Phase I clinical trials. A rapid, sensitive and specific method using LC/MS/MS has been developed for the quantitation of BPU in human plasma to perform pharmacokinetic (PK) and pharmacodynamic (PD) studies of BPU administered orally once a week. BPU is extracted from plasma into acetonitrile-*n*-butylchloride and separated on a Waters X-Terra™ MS C18 (50 × 2.1 mm, 3.5 μm) column with acetonitrile/water mobile phase (80:20, v/v) containing 0.1% formic acid using isocratic flow at 0.15 ml/min for 5 min. The analyte of interest was monitored by tandem-mass spectrometry with electrospray positive ionization with a cone voltage 15 V for BPU and 30 V for the internal standard, paclitaxel. The detector settings allowed the monitoring of the $[MH]^+$ ion of BPU (*m*/*z* 470.3) and the $[MH]^+$ of internal standard paclitaxel (*m*/*z* 854.5), with subsequent monitoring of the product ions of BPU (*m*/*z* 148.0) and paclitaxel (*m*/*z* 286.1). Calibration curves were generated over the range of 0.05–10 ng/ml with values for coefficient of determination of >0.99. The values for precision and accuracy were <20 and $\leq 15\%$, respectively. Following administration of BPU 5 mg as a weekly oral dose to a patient with advanced solid tumor malignancies, the maximum plasma concentration was 6.5 ng/ml and concentrations were quantifiable up to 173 h after administration. The lower limit of quantitation (LLOQ) of 0.05 ng/ml allows for successful measurement of plasma concentrations in patients receiving therapy with BPU as a once weekly oral dose.

Keywords: Dimethyl benzoylphenyl urea (BPU); LC/MS/MS; Pharmacokinetics

1. Introduction

Dimethyl benzoylphenyl urea (*N*-[4[(5-bromo-2-pyrimidoxyl)-3-methylphenyl]-(2-dimethylamino)-benzoylurea, BPU) is a poorly water-soluble

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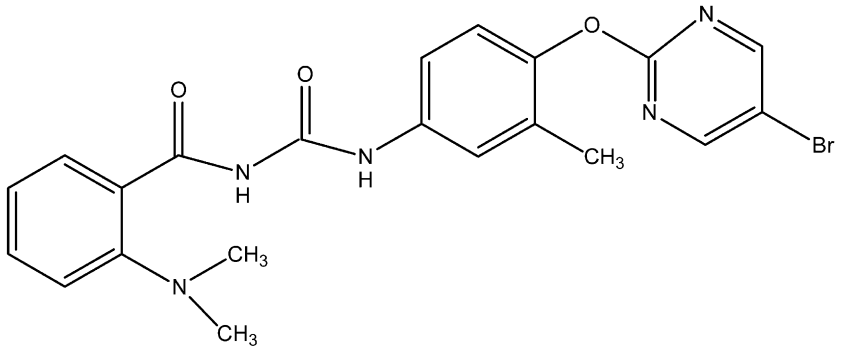

Fig. 1. Chemical structure of BPU.

benzoylphenyl urea derivative (Fig. 1) [1]. BPU and other benzoylphenyl urea derivatives inhibit tubulin polymerization, cause microtubule depolymerization in vitro and demonstrate activity against solid tumors [2]. In vitro, BPU does not inhibit DNA polymerase α while other short side-chain derivatives are able to inhibit this enzyme that is involved in DNA replication [3]. This mechanism of action has led to the clinical development of BPU as an anticancer agent.

The solubility of BPU in water is 0.00003 mg/ml [1]. This benzoylphenyl urea derivative has a partition coefficient in octanol:water of 6.21 and a pK_a of 5.0 [1]. The solubility of BPU is increased at a lower pH (pH of 1 or 2) in a variety of cosolvents, surfactants, and complexants to maximum solubility of 36.5 mg/ml at pH 1 in 20% sulfobutyl ether β-cyclodextrin [1]. For clinical use, BPU is formulated as a hard gelatin capsule containing polyglycolyzed glycerides, polyethylene glycol, and 5 mg of BPU.

BPU is currently in Phase I clinical trials in patients with refractory metastatic cancers. To characterize the clinical pharmacology, a method for the quantification of BPU in plasma was necessary. The method reported in this paper utilizes LC/MS/MS to achieve a rapid, sensitive, and specific assay method in plasma of patients receiving BPU.

2. Experimental

2.1. Chemical and reagents

BPU (NSC 639829) was a gift from the Developmental Therapeutics Program, Cancer Therapy Evaluation Program, National Institute of Health (Bethesda, MD, USA). The internal standard, paclitaxel, was supplied by Sigma Chemical Co. (St. Louis, MO, USA). Methanol (HPLC Grade) and formic acid (88%, v/v in water) were purchased from J.T. Baker (Phillipsburg, NJ, USA). Acetonitrile (HPLC grade) was obtained from EM Science (Gibbstown, NJ, USA), and *n*-butyl chloride from Allied Signal Inc. (Muskegon, MI, USA). Deionized water was obtained from a Milli-Q-UF system (Millipore, Milford, MA, USA) and used throughout in all aqueous solutions. Drug-free (blank) human plasma originated from Pittsburgh Blood Plasma, Inc. (Pittsburgh, PA, USA).

2.2. Calibration curves

Stock solution of BPU was prepared by dissolving 10 mg, accurately weighed, in 10 ml of methanol, and stored in a glass vial at −20 °C. One of the stock solutions was diluted further in blank human plasma on each day of analysis to prepare calibration samples containing BPU at concentrations of 0.05, 0.1, 0.2, 0.5, 1, 2, 5 and 10

ng/ml. Calibration curves were computed using the ratio of the peak area of BPU and internal standard by using a weighted (1/[nominal BPU concentration]) linear regression analysis. Selection of the 1/x weighting option was based on assessment of the correlation coefficient and accuracy of the standards at the lower and upper end of the calibration curve. The assay lower limit of quantitation (LLOQ) was determined to be 0.05 ng/ml. The LLOQ was determined by meeting the following two criterion: (1) the signal to noise ratio larger than 5 and (2) the values for precision and accuracy were less than 20%.

2.3. Sample preparation

Prior to extraction, frozen plasma samples were thawed in a water bath at ambient temperature. A 1-ml aliquot of plasma was added to a screw-cap glass tube (16 × 125 mm) containing 5 ml of a mixture of acetonitrile and *n*-butyl chloride (1:4, v/v) and 10 μl of a methanolic solution of paclitaxel (10 μM), used as internal standard. The tube was capped and mixed vigorously for 10 s on a vortex-mixer, and for 5 min on automated multi-tube shaker, followed by centrifugation at 2000 × *g* for 10 min at ambient temperature. The top organic layer was transferred to a disposable borosilicate glass culture tube (13 × 100 mm) and was evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residue was redissolved in 200 μl of methanol–acetonitrile–water (40:40:20, v/v/v) by vortex mixing (10 s) and immersion in an ultrasound bath (5 min). The sample was transferred to a 250-μl polypropylene autosampler vial, and a volume of 15 μl was injected onto the HPLC instrument for quantitative analysis using a temperature-controlled autosampling device operating at 15 °C.

2.4. Chromatographic and mass-spectroscopic conditions

Chromatographic analysis was performed using a Waters Model 2690 separations system (Milford, MA, USA) equipped with a Waters Model 996 photodiode-array detector. Separation of the analytes from potentially interfering material was achieved at ambient temperature using Waters X-Terra MS column (50 × 2.1 mm i.d.) packed with a 3.5-μM ODS stationary phase, protected by a guard column packed with 3.5 μm RP18 material (20 × 2.1 mm i.d.) (Milford, MA, USA). The mobile phase used for the chromatographic separation was composed of acetonitrile–water (80:20, v/v) containing 0.1% formic acid, and was delivered isocratically at a flow rate of 0.15 ml/min. The column effluent was monitored using a Micromass Quattro LC triple-quadrupole mass-spectrometric detector (Beverly, MA, USA). The instrument was equipped with an electrospray interface, and controlled by the MASSLYNX version 3.4 software (Micromass), running under Microsoft WINDOWS NT on a Compaq AP200 Pentium III computer. The samples were analyzed using an electrospray probe in the positive ionization mode operating at a cone voltage of 15 V for BPU and 30 V for paclitaxel, the internal standard. Samples were introduced into the interface through a heated nebulizer probe (350 °C). The spectrometer was programmed to allow the $[MH]^+$ ion of BPU at *m*/*z* 470.3 and that of the internal standard at *m*/*z* 854.5 to pass through the first quadrupole (Q1) and into the collision cell (Q2) (Fig. 2A and C). The collision energy was set at 35 eV for BPU and at 20 eV for the internal standard. The product ions for BPU (*m*/*z* 148.0) and the internal standard (*m*/*z* 286.1) were monitored through the third quadrupole (Q3) (Fig. 2B and D). Argon was used as collision gas at a pressure of 0.0027 mbar, and the dwell time per channel was 0.5 s for data collection.

2.5. Method validation

Method validation runs were performed on 4 consecutive days using freshly prepared samples, and included a calibration curve processed in duplicate, and quality control (QC) samples, at four different concentrations, in quadruplicate. QC samples were prepared independently in blank plasma at BPU concentrations of 0.05 (LLOQ), 0.15, 0.8 and 8 ng/ml. The accuracy and precision of the assay were assessed by the mean relative percentage deviation (DEV) from the nominal concentrations and the within-run and between-

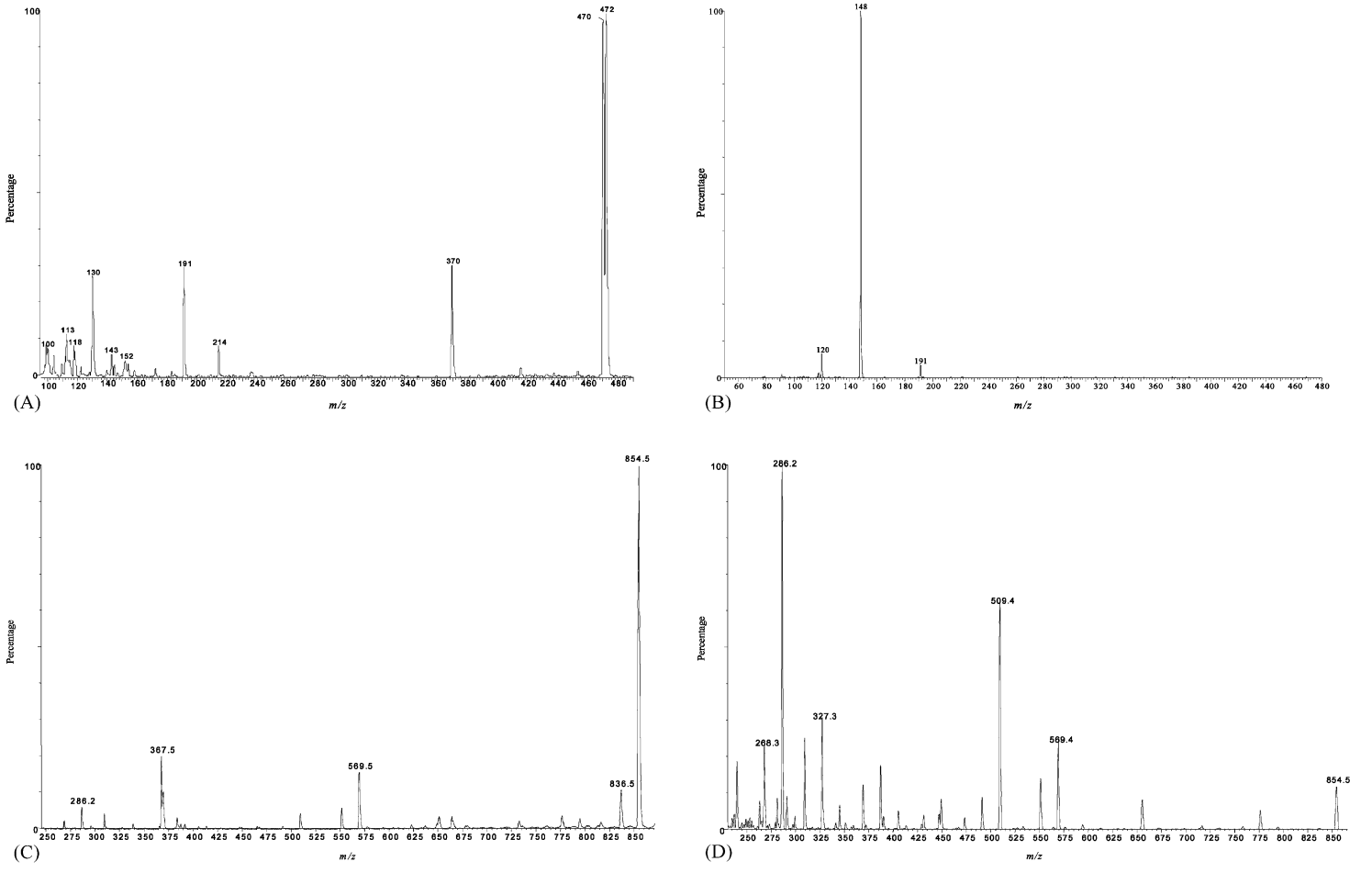

Fig. 2. (A) Full-scan positive ion Q1 spectrum showing the protonated molecule for BPU (m/z 470.3) and (B) full-scan product ion spectrum of m/z 470.3 showing a major fragment at m/z 148.0. (C) Full-scan positive ion Q1 spectrum showing the protonated molecule for paclitaxel (m/z 854.5) and (D) full-scan product ion spectrum of m/z 854.5 showing a major fragment at m/z 286.1.

run precision, respectively. The accuracy for each tested concentration was calculated as:

$$\mathrm{DEV} = 100 \times \{([\mathrm{BPU}]_{\mathrm{mean}} - [\mathrm{BPU}]_{\mathrm{nominal}})/[\mathrm{BPU}]_{\mathrm{nominal}}\}$$

Estimates of the between-run precision (BRP) were obtained by one-way analysis of variance (ANOVA) using the run day as the classification variable. The between-groups mean square (MS_{bet}), the within-groups mean square (MS_{wit}), and the grand mean (GM) of the observed concentrations across runs were calculated using the JMP™ statistical discovery software version 4 (SAS Institute, Cary, NC, USA). The BRP, expressed as a percentage relative standard deviation, was defined as:

$$\mathrm{BRP} = 100 \times (\sqrt{((\mathrm{MS}_{\mathrm{bet}} - \mathrm{MS}_{\mathrm{wit}})/n)}/GM)$$

where n represents the number of replicate observations within each run. For each concentration, the estimate of the within-run precision (WRP) was calculated as:

$$\mathrm{WRP} = 100 \times (\sqrt{(\mathrm{MS}_{\mathrm{wit}})}/GM)$$

The specificity of the method was tested by visual inspection of chromatograms of extracted human plasma samples from six different donors for the presence of interfering peaks. The extraction efficiency of the assay was measured by comparison of extracted plasma samples and aqueous samples of BPU at concentrations of 0.05, 0.15, 0.8 and 8 ng/ml. The stability of BPU in plasma was tested at the concentrations of QC samples by three freeze–thaw cycles at $-20\,^{\circ}\mathrm{C}$. The short-term stability of BPU in plasma was also assessed on the benchtop for 3 h at room temperature. Stability experiments were performed in duplicate.

2.6. Patient samples

The patient studied participated in a clinical Phase I study of BPU with dose escalation commencing at 5 mg administrated orally once weekly. The drug was formulated as 5 mg capsule containing polyglycolyzed glycerides and polyethylene glycol. The protocol was approved by the Institutional Review Board of The Johns Hopkins University School of Medicine (Baltimore, MD, USA), and the patient provided written informed consent.

Blood samples were collected in heparin-containing tubes before drug administration and at 0.25, 0.5, 1, 1.5, 2, 4, 6, 8, 24, 48, 72 and 168 h after administration of the dose. Samples were processed immediately by centrifugation for 10 min at $3000 \times g$ at ambient temperature. Plasma supernatant was collected and stored frozen at $-70\,^{\circ}\mathrm{C}$ until subsequent analysis.

BPU plasma concentrations were fit with a two-compartment model as implemented in the computer software ADAPT II (University of Southern California, Los Angeles, CA).

3. Results

3.1. Analytical procedure

The mass spectrum of BPU showed a protonated molecular ion ($[MH^+]$) at m/z 470.3 (Fig. 2A). The ion at m/z 472 is a typical protonated ion of Br ($[M+2^+]$). The high collision energy gave one major product at m/z 148.0, which was selected for subsequent monitoring in the third quadrupole (Fig. 2B). The mass spectrum of paclitaxel showed a protonated molecular ion ($[MH^+]$) at m/z 854.5 (Fig. 2C). The high collision energy gave one major product at m/z 286.1, which was selected for subsequent monitoring in the third quadrupole (Fig. 2B).

The retention times for BPU and internal standard under the optimal concentrations were 2.55 ± 0.03 and 1.96 ± 0.03 min, respectively, with an overall chromatographic run time of 5 min and a throughput time of 6 min between injections.

3.2. Method validation

Representative chromatograms of blank and spiked human plasma samples are shown in Fig. 3. The selectivity for the analysis is shown by the sharp and symmetrical resolution of the peaks, with no significant interfering peak for both BPU and internal standard in drug-free specimens, which were obtained from six different individuals.

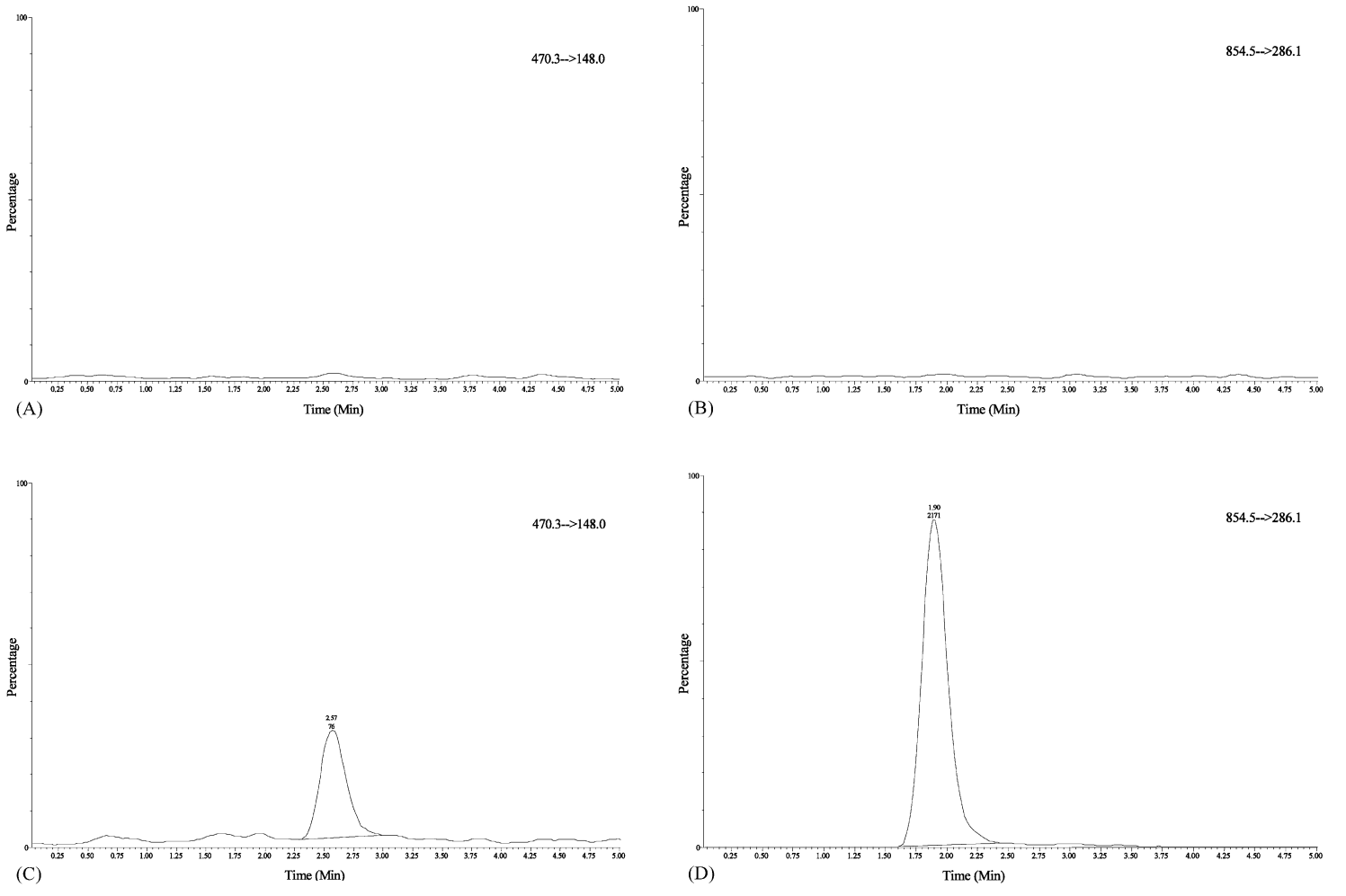

Fig. 3. Selected ion chromatograms of blank plasma (A, B), plasma spiked with 0.05 ng/ml BPU (C), and plasma spiked with 50 ng/ml of paclitaxel, the internal standard (D). The following mass-to-charge (m/z) were monitored 470.3–148.0 for BPU (A, C) and 854.5–286.1 for paclitaxel (B, D).

Calibration curves of BPU in human plasma over the range of 0.05–10 ng/ml were best fitted using a linear regression analysis, applying the peak area in combination with a weight factor (1/[nominal BPU concentration]). Heterocedasticity was observed using a linear standard curve, and therefore, a weighting factor of (1/[nominal BPU concentration]) was necessary. The correlation coefficients for the calibration curves of ≥ 0.99 were observed throughout the validation. The LLOQ was established at 0.05 ng/ml with a signal to noise ratio of 7. At this concentration, the values for accuracy, within-run, and BRP were 115, 9.37 and 3.98%, respectively. The within-run and between-run variability, expressed as the percentage relative standard deviations, were always less than 20%, whereas the mean predicted concentration was within 15% of the nominal value at the three concentrations analyzed (Table 1).

By comparing the peak area ratios of BPU containing plasma samples with those for non-processed samples prepared in the mobile phase, the mean extraction efficiency was found to be greater than 80% (Table 1). In addition, no carry-over was noted for either BPU or the internal standard.

Stock solution stored at room-temperature was stable with less than 10% degradation noted up to 6 h when raw area counts were compared with time zero. BPU is also stable at $-20\,^{\circ}C$ for up to 125 days without any significant degradation in raw area counts from time zero. There appeared to be no evidence of significant ($<10\%$) analyte degradation in human plasma at room temperature for 2 h (Table 1). Freeze–thaw stability tests were performed in duplicate and assessed through three full cycles of freeze–thaws with nominal concentrations within 20, 23 and 94% at the low, medium and high concentration, respectively. These experiments were repeated in triplicate using an HPLC method with UV detection [6] at concentrations of 0.15 and 8.0 μg/ml; nominal concentrations were less than 7% at both concentrations after two cycles of freeze–thaw, but after the third freeze–thaw cycle, were 27 and 22% of nominal at the low and high concentrations, respectively. Autosampler

Table 1
Validation characteristics of BPU in human plasma

Nominal concentration (ng/ml)	0.05	0.15	0.80	8.0
Accuracy (%)	115.0	106.7	101.5	111.0
Precision (%)				
Within-run	9.4	19.2	12.3	11.4
Between-run	4.0	11.1	[a]	8.1
Extraction recovery (%)		80.4	92.7	94.1
Stability				
Nominal concentration (ng/ml)				
Freeze–thaw cycles				
0		0.15	0.80	8.0
1		0.14	0.63	14.6
2		0.12	0.92	13.2
3		0.15	0.98	15.5
Room temperature				
Initial		0.15	ND[b]	8.0
2 h		0.14	ND[b]	7.8
Autosampler stability				
Initial		0.15		8.0
5.4 h		0.16	ND[b]	9.3

[a] No significant additional variation was observed as a result of performing the assay in different runs.
[b] ND, not done.

stability testing showed that the analyte could last 5.4 h without any degradation allowing for up to 64 samples to be analyzed within a run.

3.3. Plasma concentration–time profile

The suitability of the developed method for clinical use was demonstrated by the determina-

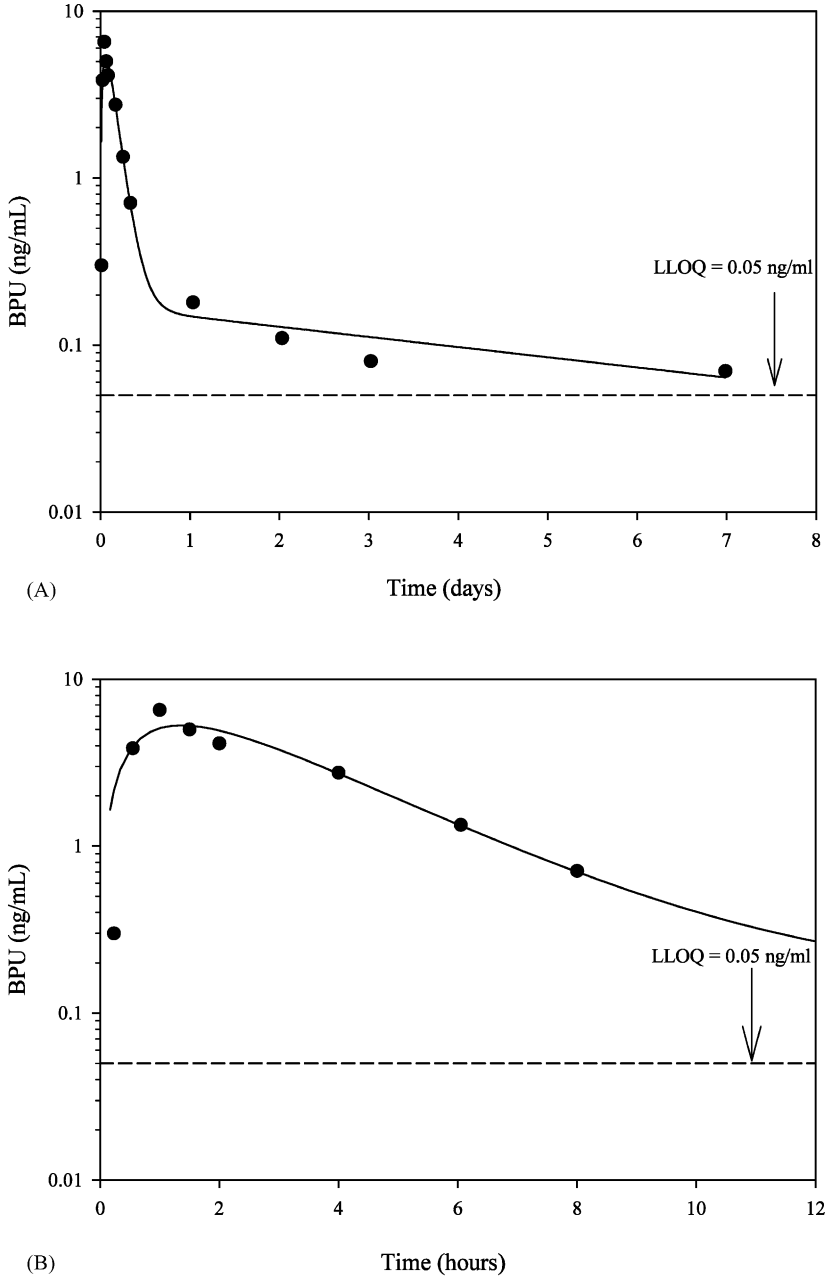

Fig. 4. Plasma concentration time curve for BPU administered orally at a dose of 5 mg to 7 days (A) and an insert of the first 12-h (B). The curves represent simulated concentration–time curves from the fit of a two-compartmental model to the data.

tion of BPU in plasma samples from a patient treated with BPU at a dose level of 5 mg. The maximum plasma concentration obtained after a single dose was 6.5 ng/ml, which occurred at 1.0 h, and concentrations were measurable out to 173 h post-treatment before administration of the next BPU dose (Fig. 4).

4. Discussion

Since BPU is a poorly water-soluble compound, a liquid–liquid extraction method was our first choice of sample preparation; this gave clean extracts free from interference. Sample pretreatment was also evaluated by a single protein precipitation step with either methanol or acetonitrile, with direct injection into the HPLC system. However, this resulted in lower response due to ion suppression. Solid phase extraction (Bond Elut, Harbor City, CA, USA) was also examined as a sample clean-up method with highly variable results (data not shown). By utilizing liquid–liquid extraction, which was developed initially for the quantitative determination of docetaxel and paclitaxel in human plasma samples, an internal standard with high extraction efficiency for the current BPU analysis was easily determined [4].

For the chromatography, the high percent organic of the eluent allowed significant retention of the analyte (2.6 min, $K' = 3.18$) and internal standard (2.0 min, $K' = 2.18$) while also giving a high sample throughput. In the final procedure, only a small fraction of the sample after extraction was injected (i.e. 15 μl of 100 μl used for reconstitution) on the column to maintain high efficiency and resolution, therefore, compromising assay sensitivity. The LLOQ of 0.05 ng/ml was sufficient to quantitate BPU concentrations in plasma following administration of the lowest dose level (5 mg) in a phase I trial. Although increased injection volumes could achieve higher response factors, overloading the column resulted in various kinds of distorted separation artifacts, including asymmetric sample bands.

In conclusion, we have developed and validated a novel assay for measuring BPU levels in human plasma. The method was validated according to the FDA bioanalytical guidelines and did not meet the acceptance criteria for precision for the low QC (0.15 ng/ml) [5]. The described method permits the analysis of patient samples to concentrations of 0.05 ng/ml, which is sufficiently sensitive to allow pharmacokinetic (PK) monitoring after oral administration of BPU at low doses. The method is currently being used to measure BPU concentrations to fully characterize the clinical pharmacology of this agent.

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