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Journal of Chromatography B, 828 (2005) 41–54
JOURNAL OF CHROMATOGRAPHY B
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Validation and implementation of a liquid chromatography/tandem mass spectrometry assay to quantitate dimethyl benzoylphenylurea (BPU) and its five metabolites in human plasma and urine for clinical pharmacology studies

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Received 24 May 2005; accepted 4 September 2005
Available online 25 October 2005

Abstract

A method has been developed for the quantitation of *N*-[4-(5-bromo-2-pyrimidinyloxy)-3-methylphenyl]-*N*′-(2-dimethylamino-benzoyl)urea (BPU) and its metabolites in human plasma and urine. BPU and metabolites were separated on a C18 column with acetonitrile–water mobile phase containing 0.1% formic acid using isocratic flow for 5 min. The analytes were monitored by tandem mass spectrometry. Calibration curves were generated over the range of 2.5–500 ng/mL for BPU, mmBPU, and aminoBPU in plasma; and 0.1–20, 0.1–20, 0.5–100, 10–2000, 1–200, and 3–600 ng/mL for BPU, mmBPU, aminoBPU, G280, G308, and G322 in urine, respectively. The method has been successfully applied to study the pharmacokinetics of BPU.

Keywords: Dimethyl benzoylphenylurea (BPU); LC/MS/MS; Pharmacokinetics

1. Introduction

Benzoylphenylureas were initially developed as insecticides [1,2]. *N*-[4-(5-Bromo-2-pyrimidinyloxy)-3-chlorophenyl]-*N*′-(2-nitrobenzoyl)urea (HO-221) was the lead benzoylphenylurea compound with noted antitumor activity but poor physico-chemical characteristics therefore limiting its potential clinical utility [2–4]. *N*-[4-(5-Bromo-2-pyrimidinyloxy)-3-methylphenyl]-*N*′-(2-dimethylamino-benzoyl)urea (BPU, NSC 639829, Fig. 1), an HO-221 analogue and poorly water-soluble benzoylphenylurea derivative, has reported cytotoxic activities [5]. The mechanism of action for benzoylphenylurea derivatives includes tubulin polymerization inhibition and microtubule depolymerization in vitro [4,6].

In murine pharmacokinetic studies, BPU was metabolized to monomethyl-BPU (mmBPU, Fig. 1) and didesmethyl-BPU (aminoBPU, NSC 647884, Fig. 1), which were shown to have in vitro cytotoxic activity similar to the parent compound with activity against murine P388 leukemia, human AIDS-related lymphoma, breast, and prostate carcinoma [4,6,7]. Bioavailability was low and variable for both a 5 mg (12–29%) and a 25 mg (4.4–26%) capsule in dogs [8]. The mechanism of action, oral formulation, and favorable anti-tumor activity in preclinical models lead to the clinical development of BPU as an anticancer agent.

BPU is currently being evaluated in phase I clinical trials in patients with refractory metastatic cancers with the drug being administered orally once weekly on a continuous schedule or for 6 out of 8 weeks [9,10]. Initially, BPU was quantitated using LC/MS/MS over the range of 0.05–10 ng/mL [11]. As dose escalation continued in the phase I clinical trial, a LC/UV method was developed to quantitate BPU in the range of 10 ng/mL–10 μg/mL [12]. Using the LC/UV and the LC/MS/MS methods, five BPU metabolites were identified in vivo in either urine or plasma from patient receiving oral BPU [11–13]. In order to comprehensively characterize the clinical

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doi:10.1016/j.jchromb.2005.09.027

pharmacology of BPU, a method for the quantitation of BPU and its metabolites in plasma and urine was necessary. BPU, mmBPU, and aminoBPU were quantitated in a clinically relevant range in plasma and urine, while BPU's three non-cytotoxic metabolites (G280, G308, and G322) were quantitated in urine. G280, G308, and G322 were not assessed in plasma since initial identification of these metabolites was limited to urine samples. The assay reported in this paper utilizes LC/MS/MS to achieve a rapid, sensitive, and specific method in plasma and urine of patients receiving BPU.

2. Experimental

2.1. Chemical and reagents

BPU (NSC 639829) and aminoBPU (NSC 647884) were a gift from the Developmental Therapeutics Program, Cancer Therapy Evaluation Program, National Institute of Health (Bethesda, MD, USA). mmBPU, G280, G308, and G322 were synthesized in the Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins Medicinal Chemistry Core (Baltimore, MD, USA). The internal standard, temazepam, was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Formic acid (88%, v/v in water) was purchased from J.T. Baker (Phillipsburg, NJ, USA) and *n*-butyl chloride from Honeywell, Burdick & Jackson (Muskegon, MI, USA). Acetonitrile and methanol were HPLC Grade and were obtained from EM Science (Gibbstown, NJ, USA). Deionized water was obtained from a Milli-Q-UF system (Millipore, Milford, MA, USA) and used in all aqueous solutions. Drug-free (blank) human plasma originated from Pittsburgh Blood Plasma Inc. (Pittsburgh, PA, USA). Human urine was obtained from healthy volunteers that were willing to donate urine.

2.2. Preparation of stock solutions

Stock solutions of BPU, mmBPU, G280, G308, and G322 were prepared in duplicate at 0.1 mg/mL by dissolving 2 mg, accurately weighed, in 20 mL of methanol. The stock solution of aminoBPU was prepared in duplicate at 0.01 mg/mL by dissolving 2 mg, accurately weighed, in 200 mL of methanol. The area counts for each of the duplicated aliquots were checked in quintuplicate, and if the mean value for area counts was within 5%, the stock solutions were then stored in a glass vial at −20 °C. Stock solutions of BPU, mmBPU, aminoBPU, and G280 were stable for 3, 4, 4, and 4 months, respectively.

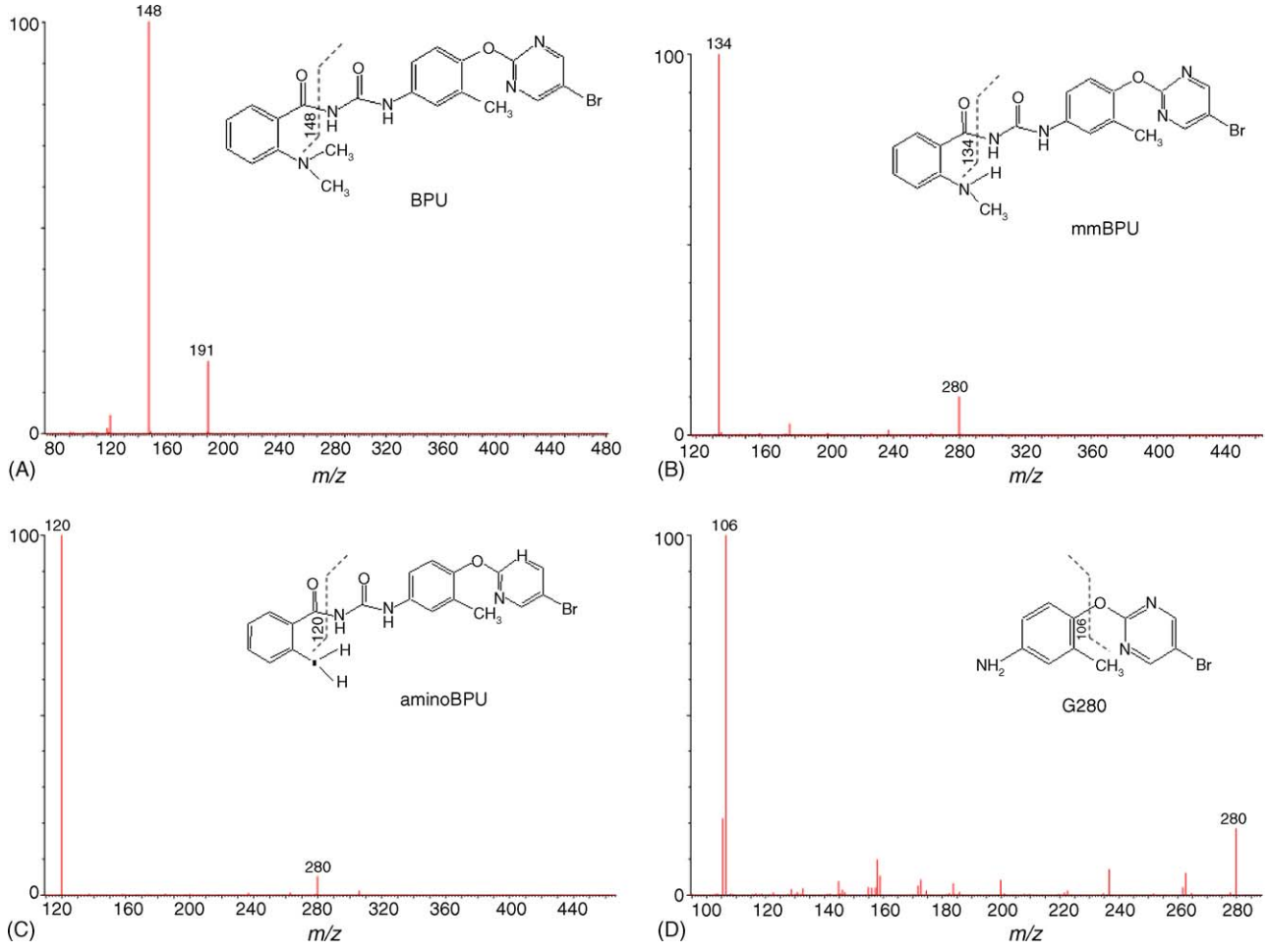

Fig. 1. Full-scan product ion spectrum and chemical structure for BPU (A), mmBPU (B), aminoBPU (C), G280 (D), G308 (E), G322 (F), and temazepam (G).

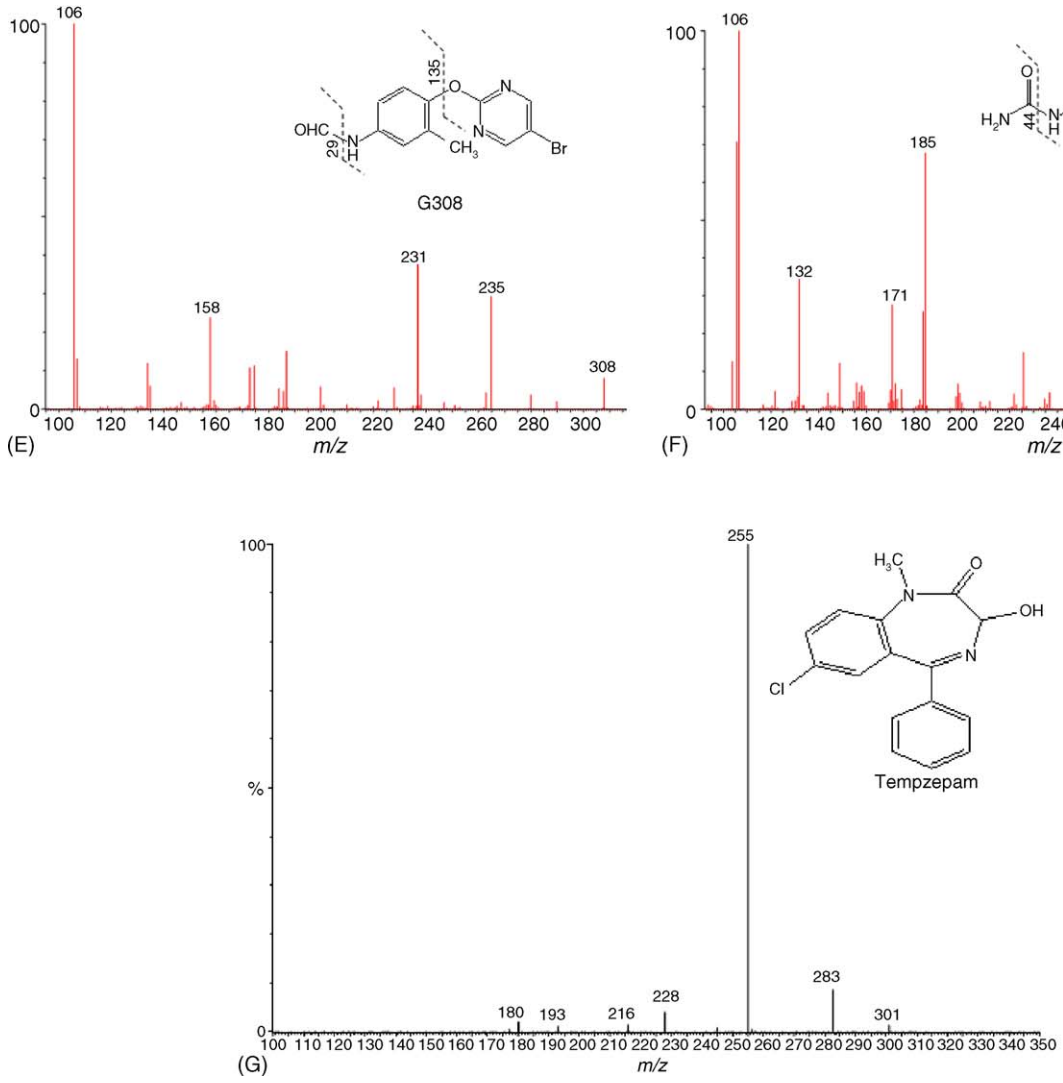

Fig. 1. (*Continued*).

A stock solution of temazepam was prepared by dissolving 10 mg, accurately weighed, in 10 mL of methanol, with further dilution to 0.1 mg/mL with methanol. The 0.1 mg/mL solution was stored in a glass vial at −20 °C for 1 month.

2.2.1. Preparation of calibration standards and quality controls in plasma

BPU, mmBPU, and aminoBPU stock solutions were diluted in acetonitrile:water (1:1, v/v) on each day of analysis to spike into pooled human plasma to prepare calibration curve standards and quality control (QC) samples. Eight standards were prepared including the concentrations 2.5, 5, 12.5, 25, 50, 125, 250, and 500 ng/mL, and four QC were prepared at concentrations 2.5 (lower limit of quantitation (LLOQ)), 3.75, 37.5, and 375 ng/mL. After samples obtained from patients were analyzed, it was determined a dilutional QC was necessary. This QC was prepared at 2500 ng/mL and diluted 1:5 and 1:10 in pooled human plasma for quantitation. All standards and QC samples were prepared fresh daily. For long-term and freeze–thaw stability, QC samples were stored at −70 °C.

A 50 μL aliquot of the 0.1 mg/mL internal standard stock solution was added into 100 mL of acetonitrile for a final concentration of 50 ng/mL at the time of analysis for plasma samples.

2.2.2. Preparation of calibration standards and quality controls in urine

BPU, mmBPU, aminoBPU, G280, G308, and G322 stock solutions were diluted in acetonitrile:water (1:1, v/v) to spike into pooled human urine to prepare a calibration curve with seven standards and four QC samples (Table 1). All standards and quality controls were prepared fresh daily. For long-term stability, quality controls were stored at −70 °C. Freeze–thaw stability was not performed with urine since a sufficient number of aliquots were made with the patient samples to allow for only the initial thaw.

A 10 μL aliquot of the 0.1 mg/mL internal standard stock solution was added into 1000 mL of *n*-butyl chloride for a final

Table 1
Final concentrations of urine calibrators and QC samples

Calibrators	BPU (ng/mL)	mmBPU (ng/mL)	aminoBPU (ng/mL)	G280 (ng/mL)	G308 (ng/mL)	G322 (ng/mL)
1 (LLOQ)	0.1	0.1	0.5	10	1	3
2	0.2	0.2	1	20	2	6
3	0.5	0.5	2.5	50	5	15
4	1	1	5	100	10	30
5	5	5	25	500	50	150
6	10	10	50	1000	100	300
7	20	20	100	2000	200	600
QCs						
LLOQ	0.1	0.1	0.5	10	1	3
Low	0.15	0.15	0.75	15	1.5	4.5
Medium	0.7	0.7	3.5	70	7	21
High	18	18	90	1800	180	540

concentration of 1 ng/mL at the time of analysis for urine samples.

2.3. Sample preparation

2.3.1. Plasma

Prior to extraction, frozen plasma samples were thawed in a water bath at ambient temperature. A 100 μL aliquot of plasma was added to a borosilicate glass tube (13 mm × 100 mm) followed by 300 μL of 50 ng/mL temazepam in acetonitrile. The tube was mixed vigorously for 30 s on a vortex-mixer followed by centrifugation at 1200 × *g* for 10 min at ambient temperature. The top organic layer was transferred to a 250 μL polypropylene autosampler vial, and a volume of 15 μL was injected onto the LC/MS/MS instrument for quantitative analysis using an autosampling device.

2.3.2. Urine

Prior to extraction, frozen urine samples were thawed in a water bath at ambient temperature. A 1 mL aliquot of urine was added to a screw-cap glass tube (16 mm × 125 mm) containing 5 mL of a mixture of 1 ng/mL temazepam in *n*-butyl chloride. The tube was capped and mixed vigorously for 30 s on a vortex-mixer, and for 5 min on automated multi-tube shaker, followed by centrifugation at 1200 × *g* for 10 min at ambient temperature. The top organic layer (4 mL) was transferred to a disposable borosilicate glass culture tube (13 mm × 100 mm) and was evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residue was redissolved in 100 μL of acetonitrile–water (50:50, v/v) by vortex mixing (30 s) and immersion in an ultrasound bath (3 min). The sample was transferred to a 250 μL polypropylene autosampler vial, and a volume of 15 μL was injected onto the LC/MS/MS instrument for quantitative analysis using an autosampling device.

2.4. Equipment

The equipment and conditions of the assay were identical for the plasma and urine matrix. Chromatographic analysis was performed using an Agilent 1100 series HPLC (Agilent Technologies, Palo Alto, CA, USA). Separation of the analyte from potentially interfering material was achieved at ambient temperature using Waters XTerra MS column (50 mm × 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 (Milford, MA, USA). The mobile phase used for the chromatographic separation was composed of acetonitrile–water (70:30, 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 an API 3000 triple-quadrupole mass-spectrometric detector (Applied Biosystems, Foster City, CA, USA). The instrument was equipped with an electrospray interface, operated in a positive mode and controlled by the Analyst version 1.2 software (Applied Biosystems). Samples were introduced into the interface through a heated nebulizer probe set at 350 °C. A high voltage of 5.2 kV was applied to the ion spray. The settings of nebulizer gas, curtain gas, and collision gas were 10, 9, and 8 psi, respectively. Other optimal parameters included declustering potential (DP), focusing potential (FP), entrance potential (EP), collision energy (CE), and collision cell exit potential (CXP) and are reported in Table 2. The spectrometer was programmed in multiple reaction monitoring (MRM) mode to allow the specific transition of precursor ion to fragment for each compound (Fig. 1). The dwell time per channel was 150 ms for data collection.

2.5. Calibration curves

2.5.1. Plasma

BPU, mmBPU, and aminoBPU calibration samples were prepared in plasma over the range of 2.5–500 ng/mL. Calibration

Table 2
Optimization parameters for BPU and metabolites

	DP (V)	FP (V)	EP (V)	CE (V)	CXP (V)
BPU	11	170	10	47	8
mmBPU	11	190	10	45	10
aminoBPU	16	180	10	29	6
G280	61	210	10	31	8
G308	51	260	10	31	8
G322	26	250	10	47	6
Temazepam	36	170	10	33	16

curves were computed using the ratio of the peak area of the analyte and internal standard by using a weighted (1/[nominal concentration]) linear regression analysis. The parameters of each calibration curve were used to compute back-calculated concentrations and to obtain values for the QC samples and unknown samples by interpolation.

2.5.2. Urine

BPU, mmBPU, aminoBPU, G280, G308, and G322 calibration samples were prepared in urine over the range of 0.1–20, 0.1–20, 0.5–100, 10–2000, 1–200, and 3–600 ng/mL, respectively. Calibration curves were computed using the ratio of the peak area of the analyte and internal standard by using a weighted (1/[nominal concentration]) linear regression analysis, except for G280 which used a weighted (1/[nominal concentration]) quadratic equation. The parameters of each calibration curve were used to compute back-calculated concentrations and to obtain values for the QC samples and unknown samples by interpolation.

2.6. Validation procedures

2.6.1. Plasma

2.6.1.1. Pre-study validation. Method validation runs were performed on 4 days. Each analytical run consisted of a calibration curve using single standards at each concentration except duplicate standards were analyzed for the LLOQ, upper limit of quantitation (ULQ), and QC samples. Dilutional QC samples were performed on at least three separate occasions in triplicate. 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-run precision, respectively. The accuracy for each tested concentration was calculated as:

$$\mathrm{DEV}_{(\mathrm{analyte})} = 100 \times \left\{ \frac{[\mathrm{analyte}]_{\mathrm{mean}} - [\mathrm{analyte}]_{\mathrm{nominal}}}{[\mathrm{analyte}]_{\mathrm{nominal}}} \right\}$$

Estimates of the between-run precision were obtained by one-way analysis of variance (ANOVA) using the run day as the classification variable. The between-groups mean square ($\mathrm{MS_{bet}}$), the within-groups mean square ($\mathrm{MS_{wit}}$), 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 between-run precision (BRP), expressed as a percentage relative standard deviation, was defined as:

$$\mathrm{BRP} = 100 \times \left(\frac{\sqrt{(\mathrm{MS_{bet}} - \mathrm{MS_{wit}})/n}}{\mathrm{GM}} \right)$$

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:

$$\mathrm{WRP} = 100 \times \left(\frac{\sqrt{\mathrm{MS_{wit}}}}{\mathrm{GM}} \right)$$

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 endogenous or exogenous interfering peaks. The peak area needed to be less than 20% than the peak area for BPU, mmBPU, and aminoBPU at 2.5 ng/mL in an aqueous solution. If not, plasma from six additional donors would be tested. The relative recovery of the analytes was measured by comparison of the mean concentration values of extracted plasma samples in triplicate and aqueous samples at concentrations of the low and high QC samples. Autosampler stability was also assessed at concentrations of the low and high QC samples with continuous injection of samples for approximately 9 h. The long-term stability test was assessed at concentrations of the low and high QC samples in triplicate at −70 °C at 138 days. The mean values of the triplicate samples were compared to the initial condition for long-term stability.

Crossvalidation with the previously described LC/MS/MS assay was performed by assessing the precision and accuracy of BPU at 5 and 150 ng/mL, which was diluted 1:100 for quantitation using the original LC/MS/MS method [11]. For crossvalidation, samples were prepared independently in blank human plasma in five replicates on three separate days and were analyzed using both analytical methods.

2.6.1.2. In-study validation. Samples from patients receiving treatment with BPU were analyzed over a time period involving 13 separate days. Each analytical run consisted of a calibration curve using single standards at each concentration except duplicate standards were analyzed for the LLOQ, ULQ, and QC samples in duplicate or triplicate if greater than 40 unknown samples were analyzed. The accuracy and precision of the assay were assessed by the same methodology as the plasma samples (see Section 2.6.1.1).

2.6.2. Urine

2.6.2.1. Pre-study validation. Method validation runs were performed on 3 days. Each analytical run consisted of a calibration curve using single standards at each concentration except duplicate standards were analyzed for the LLOQ, ULQ, and QC samples. The accuracy and precision of the assay were assessed by the same methodology as the plasma samples (see Section 2.6.1.1).

The specificity of the method was tested by visual inspection of chromatograms of extracted human urine samples from six different donors for the presence of endogenous or exogenous interfering peaks. The peak area needed to be less than 20% than the peak area for BPU (0.1 ng/mL), mmBPU (0.1 ng/mL), aminoBPU (0.5 ng/mL), G280 (10 ng/mL), G308 (1 ng/mL), and G322 (0.3 ng/mL) in an aqueous solution. If not, urine from six additional donors would be tested. The extraction efficiency of the assay was measured by comparison of the mean values of extracted urine samples in triplicate and aqueous samples at concentrations of the low and high QC samples. Autosampler stability was also assessed at concentrations of the low and high QC samples with continuous injection of samples for approximately 22 h. The long-term stability test was assessed at concentrations of the low and high QC samples in triplicate at −70 °C at 91 days. The mean values of samples processed in

triplicate were compared to the initial condition for long-term stability.

2.6.2.2. In-study validation. Patient sample runs were performed on 4 days. Each analytical run consisted of a calibration curve using single standards at each concentration except duplicate standards were analyzed for the LLOQ, ULQ, and QC samples in duplicate since less than 40 patient samples were analyzed. The accuracy and precision of the assay were assessed by the same methodology as the plasma samples (see Section 2.6.1.1).

2.7. Pharmacokinetic analysis

The patient participated in a phase I study and received a dose of BPU of 150 mg administered orally once weekly. The drug was formulated as a 25 mg capsule containing polyglycolyzed glycerides and polyethylene glycol and stored under refrigeration. The protocol was approved by the Institutional Review Board of the Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins (Baltimore, MD, USA), and the patient provided written informed consent.

2.7.1. Plasma

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, and 72 h after administration of the dose during week 1. Samples were processed immediately by centrifugation for 10 min at 3000 × *g* at ambient temperature. Plasma supernatant was split into two aliquots, and stored at −70 °C until subsequent analysis.

Pharmacokinetic parameters after a single dose of BPU on day 1 were estimated using model-independent methods as implemented in the computer software program WinNonlin (Version 3.1, Pharsight Corp.). The maximum plasma concentration (C_{max}) and the time of C_{max} after oral administration were obtained by visual inspection of the plasma concentration–time curve. The area under the plasma concentration–time curve (AUC) was calculated using the log-linear trapezoidal rule. Apparent oral clearance was calculated as dose/AUC.

2.7.2. Urine

Urine was collected continually during five collection intervals: 0–4, 4–8, 8–24, 24–48, and 48–72 h after administration of the BPU dose during week 1. Urine was collected in plastic containers and was refrigerated after collection. For each collection period, the total volume of urine was recorded and two 15 mL aliquots were frozen at −70 °C until subsequent analysis.

The cumulative amount of BPU and metabolites excreted in the urine was calculated as a product of the concentration and urine volume accumulated by day. The cumulative percent excreted in the urine was determined as the cumulative amount divided by the actual dose of BPU expressed in milligrams, assuming 100% bioavailability. A catheter was not used during the collection of urine.

3. Results and discussion

3.1. Chromatographic separation and detection

An LC/MS/MS method to quantitatively determine BPU, mmBPU, and aminoBPU concentrations in human plasma was developed, validated, and implemented to quantitate drug in plasma from patients receiving treatment with BPU. This LC/MS/MS method also quantitatively determined BPU, mmBPU, aminoBPU, G280, G308, and G322 concentrations in human urine. The chromatographic separation and detection were identical utilizing the extracted plasma or urine as the matrix. The following mass-to-charge (*m*/*z*) ratios were monitored 470.1 > 148.0 for BPU, 456.1 > 134.0 for mmBPU, 442.1 > 128.0 for aminoBPU, 280.0 > 105.9 for G280, 308.0 > 105.6 for G308, 322.8 > 105.9 for G322, and 301.0 > 254.8 for the internal standard (Fig. 1).

No peaks were observed in the chromatograms of blank plasma or urine from six donors when monitored for BPU, mmBPU, aminoBPU, G280, G308, G322, and the internal standard (data not shown). During implementation of this assay, pretreatment samples from thirteen cancer patients were analyzed with this assay with no interferences noted in the pre-sample for either plasma or urine. Representative chromatograms of blank human plasma, plasma spiked with BPU, mmBPU, aminoBPU, and an unknown plasma sample (8 h time point) from a patient that received 150 mg of BPU administered orally are shown in Fig. 2. Representative chromatograms of blank human urine, urine spiked with BPU, mmBPU, aminoBPU, G280, G308, G322, and an unknown urine sample (24–48 h urine collection period) from a patient that received 150 mg of BPU administered orally are shown in Fig. 3. The mean retention times for BPU, mmBPU, aminoBPU, G280, G308, G322, and the internal standard under the optimal conditions were 2.85, 2.65, 1.90, 1.20, 1.40, 1.25, and 3.88 min, respectively. The overall chromatographic run time was 5 min.

3.2. Linearity of detector responses

3.2.1. Plasma

Using a linear standard curve over the entire range of 2.5–500 ng/mL with a weighting factor (1/[nominal concentration]), calibration curves for BPU, mmBPU, and aminoBPU standards were constructed from the peak area ratio of the analyte to the internal standard with an excellent linear relationship ($r > 0.99$, range = 0.9983–0.9998). The weighting factor was chosen compared to uniform weighting after evaluation of goodness-of-fit by assessment of the R^2 value, intercept closest to a zero value, percent recovery of calibrators and QC samples, and assessment of residuals.

For each point on the calibration curves for BPU, mmBPU, and aminoBPU, the concentrations back-calculated from the equation of the regression analysis were always within 9.8% of the nominal value, except at the mmBPU LLOQ, where the accuracy was within 12.4% of the nominal value (Table 3). A linear regression of the back-calculated concentrations versus the nominal values provided a unit slope and an intercept not

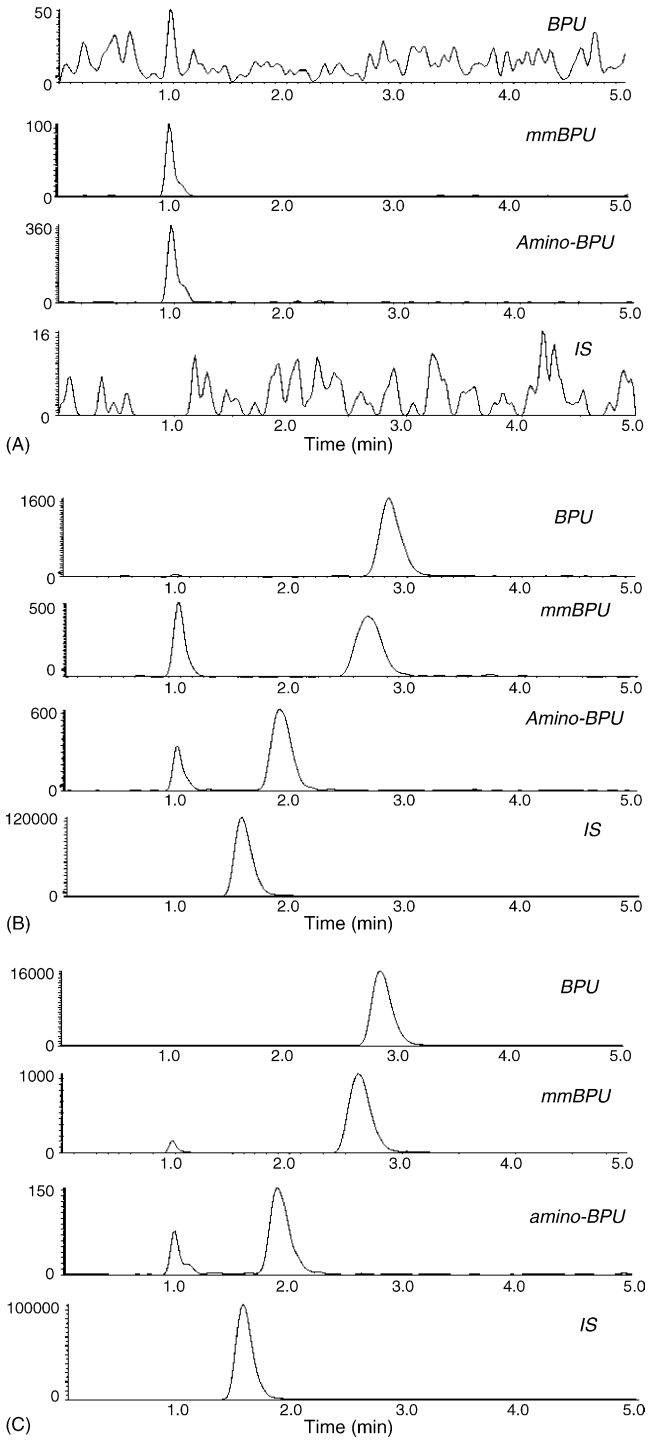

Fig. 2. Selected ion chromatograms of blank plasma (A), plasma spiked at the LLOQ concentration of BPU/mmBPU/aminoBPU with retention times of 2.85/2.65/1.90 min (B), and a select patient sample obtained 8 h after the oral administration of 150 mg of BPU (C). The following mass-to-charge (*m/z*) ratios were monitored 470.1 > 148.0 for BPU, 456.1 > 134.0 for mmBPU, 442.1 > 120.0 for aminoBPU, and 301.0 > 254.8 for internal standard.

significantly different from zero (data not shown). The distribution of the residuals showed random variation, was normally distributed, and centered on zero (data not shown).

The LLOQ for BPU, mmBPU, and aminoBPU was established at 2.5 ng/mL for human plasma, at which the

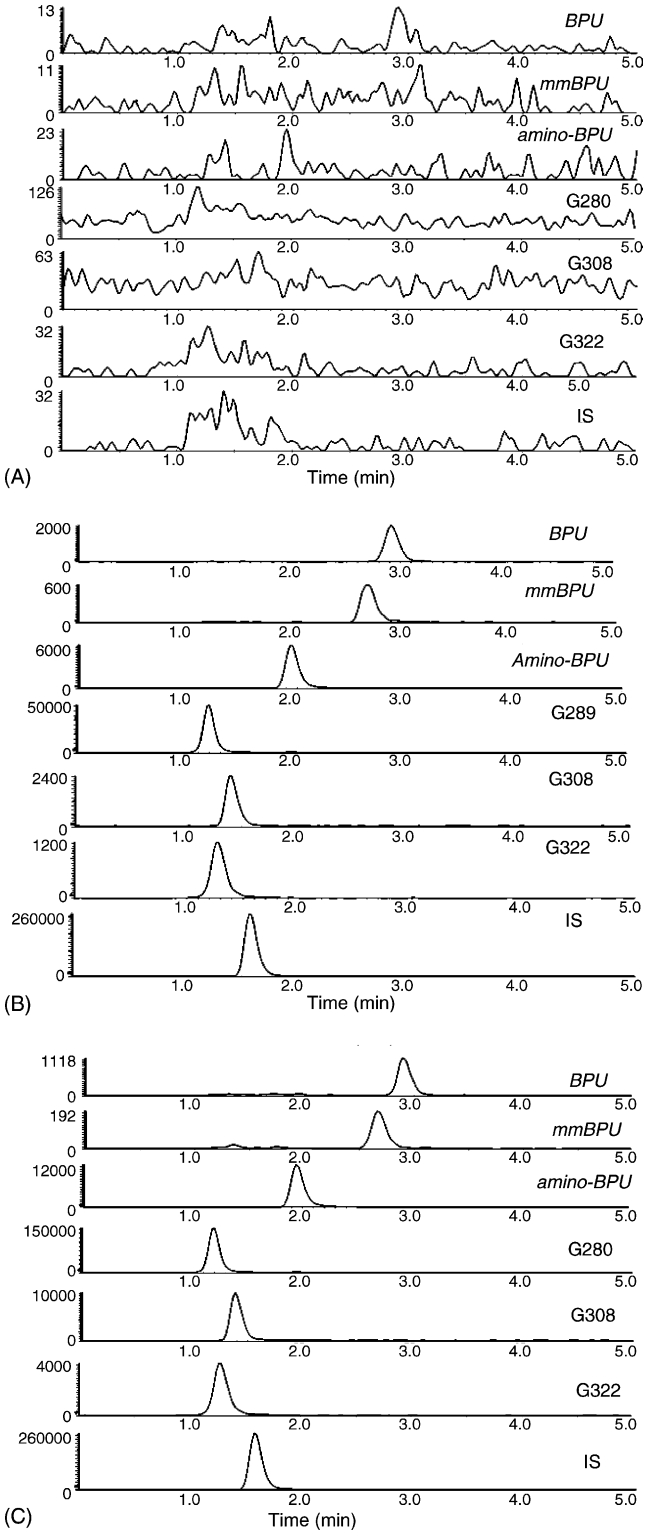

Fig. 3. Selected ion chromatograms of blank urine (A), urine spiked with BPU/mmBPU/aminoBPU/G280/G308/G322 with retention times of 2.85/2.65/1.90/1.20/1.40/1.25 min (B), and a select patient sample from the 24 to 48 h urine collection after the oral administration of 150 mg of BPU (C). The following mass-to-charge (m/z) ratios were monitored 470.1 > 148.0 for BPU, 456.1 > 134.0 for mmBPU, 442.1 > 120.0 for aminoBPU, 280.0 > 105.9 for G280, 308.0 > 105.6 for G308, 322.8 > 105.9 for G322, and 301.0 > 254.8 for internal standard.

Table 3
Back-calculated concentrations from calibration curves for BPU, mmBPU, and aminoBPU in human plasma

Nominal concentration (ng/mL)	*n*	Accuracy (%)	Concentration (ng/mL)[a]	Precision (%)	
				Within-run	Between-run
BPU					
2.5	8	100.1	2.50 ± 0.10	2.2	3.7
5	4	98.7	4.94 ± 0.13	[b]	2.6
12.5	4	102.6	12.83 ± 0.56	[b]	4.4
25	4	96.9	24.23 ± 0.81	[b]	3.3
50	4	101.9	50.93 ± 1.43	[b]	2.8
125	4	99.0	123.75 ± 7.50	[b]	6.1
250	4	101.8	254.50 ± 15.86	[b]	6.2
500	8	99.7	498.25 ± 11.72	1.9	1.5
mmBPU					
2.5	8	112.4	2.81 ± 0.22	3.8	7.4
5	4	101.7	5.08 ± 0.38	[b]	7.4
12.5	4	95.8	11.98 ± 0.64	[b]	5.3
25	4	90.2	22.55 ± 0.52	[b]	2.3
50	4	94.7	47.33 ± 2.80	[b]	5.9
125	4	91.2	114.00 ± 2.45	[b]	2.2
250	4	97.4	243.50 ± 6.66	[b]	2.7
500	8	102.3	511.38 ± 10.88	2.6	[c]
aminoBPU					
2.5	8	106.6	2.67 ± 0.15	7.0	[c]
5	4	99.6	4.98 ± 0.23	[b]	4.7
12.5	4	99.8	12.48 ± 0.05	[b]	0.4
25	4	93.1	23.28 ± 0.67	[b]	2.9
50	4	95.5	47.75 ± 1.05	[b]	2.2
125	4	97.4	121.75 ± 1.26	[b]	1.0
250	4	101.0	252.50 ± 7.72	[b]	3.1
500	8	100.5	502.38 ± 7.48	1.8	[c]

[a] Values are mean ± standard deviation.
[b] Not calculated.
[c] No significant additional variation was observed as a result of performing the assay in different runs.

concentration was associated with a mean (±standard deviation) signal-to-noise ratio of 245.3 ± 37.6, 168.3 ± 16.6, and 284.3 ± 4.0 from three observations.

3.2.2. Urine

Calibration curves for BPU, mmBPU, aminoBPU, G280, G308, and G322 standards were constructed from the peak area ratio of the analyte to the internal standard. An excellent linear relationship ($r > 0.99$, range = 0.9947–0.9999) for BPU, mmBPU, aminoBPU, G308, and G322 was observed using a linear standard with a weight factor (1/[nominal concentration]). For G280, the linear relationship ($r > 0.99$, range = 0.9990–0.9998) was observed when applying a quadratic equation with a weight factor (1/[nominal concentration]). The weighting factor was chosen as described for plasma (see Section 3.2.1).

For each point on the calibration curves for BPU, mmBPU, aminoBPU, G280, and G322, the concentrations back-calculated from the equation of the regression analysis were always within 11.1% of the nominal value, except at the G308 LLOQ, where the accuracy was within 14.8% of the nominal value (Table 4). A linear regression of the back-calculated concentrations versus the nominal values provided a unit slope and an intercept not significantly different from zero (data not shown). The distribution of the residuals showed random variation, was normally distributed, and centered on zero (data not shown).

The LLOQ for BPU, mmBPU, aminoBPU, G280, G308, and G322 was established for human urine at 0.1, 0.1, 0.5, 10, 1, and 3 ng/mL, at which the concentration was associated with a mean (±standard deviation) signal-to-noise ratio of 250.7 ± 55.2, 191.0 ± 46.8, 237.3 ± 232.7, 727.3 ± 634.5, 106.0 ± 42.8, and 116.3 ± 86.8, respectively, from three observations.

3.3. Accuracy, precision, and recovery

3.3.1. Plasma

A prior analytical method to quantitate BPU utilized liquid–liquid extraction with tandem mass spectrometric detection to achieve a lower level of sensitivity of 0.05 ng/mL in human plasma [11]. This level of sensitivity was not necessary as dose escalation continued in the phase I clinical trial and led to the development of a LC/UV method that would not require a 1:200 dilution to quantitate patient samples [12]. Using a combination of the LC/UV and the original LC/MS/MS methods, we were able to confirm the presence of five metabolites in either plasma or urine [13]. To study the complete clinical pharmacology of BPU, the LC/UV method was not specific to separate BPU and its five metabolites although this method

Table 4
Back-calculated concentrations from calibration curves for BPU, mmBPU, aminoBPU, G280, G308, and G322 in human urine

Nominal concentration (ng/mL)	*n*	Accuracy (%)	Concentration (ng/mL)[a]	Precision (%)	
				Within-run	Between-run
BPU					
0.1	6	99.7	0.10 ± 0.01	5.8	2.9
0.2	3	105.8	0.21 ± 0.02	[b]	8.2
0.5	3	98.5	0.49 ± 0.06	[b]	11.7
1	3	100.6	1.01 ± 0.08	[b]	8.0
5	3	100.1	5.00 ± 0.27	[b]	5.4
10	3	91.2	9.12 ± 0.24	[b]	2.6
20	6	102.2	20.4 ± 1.31	8.2	[c]
mmBPU					
0.1	6	106.5	0.11 ± 0.004	5.8	[c]
0.2	3	98.0	0.20 ± 0.01	[b]	4.4
0.5	3	99.5	0.50 ± 0.02	[b]	4.3
1	3	95.3	0.95 ± 0.05	[b]	5.4
5	3	94.0	4.70 ± 0.09	[b]	1.8
10	3	97.5	9.75 ± 0.17	[b]	1.7
20	6	101.4	20.3 ± 0.21	1.0	0.1
aminoBPU					
0.5	6	102.0	0.51 ± 0.05	3.6	10.1
1	3	102.2	1.02 ± 0.04	[b]	4.0
2.5	3	99.9	2.50 ± 0.23	[b]	9.4
5	3	100.0	5.00 ± 0.11	[b]	2.3
25	3	93.3	23.3 ± 3.07	[b]	13.2
50	3	98.0	49.0 ± 3.64	[b]	7.4
100	6	101.3	101 ± 3.37	1.1	3.5
G280					
10	6	100.9	10.1 ± 1.08	4.6	10.8
20	3	97.7	19.5 ± 2.05	[b]	10.5
50	3	102.5	51.3 ± 2.05	[b]	4.0
100	3	98.2	98.2 ± 14.0	[b]	14.2
500	3	98.5	493 ± 31.8	[b]	6.5
1000	3	101.7	1017 ± 32.2	[b]	3.2
2000	6	99.8	1997 ± 53.5	3.4	[c]
G308					
1	6	85.2	0.85 ± 0.04	4.6	[c]
2	3	97.5	1.95 ± 0.07	[b]	3.6
5	3	111.1	5.55 ± 0.12	[b]	2.2
10	3	110.3	11.0 ± 0.57	[b]	5.2
50	3	110.8	55.4 ± 1.95	[b]	3.5
100	3	107.3	107 ± 1.15	[b]	1.1
200	6	96.6	193 ± 2.99	1.7	[c]
G322					
3	6	97.3	2.92 ± 0.36	3.2	13.2
6	3	110.8	6.65 ± 0.08	[b]	1.1
15	3	97.1	14.6 ± 1.55	[b]	10.6
30	3	104.8	31.4 ± 3.68	[b]	11.7
150	3	92.2	138 ± 8.02	[b]	5.8
300	3	97.4	292 ± 11.7	[b]	4.0
600	6	101.5	609 ± 13.8	2.0	1.3

[a] Values are mean ± standard deviation.
[b] Not calculated.
[c] No significant additional variation was observed as a result of performing the assay in different runs.

did have a dynamic analytical range (10 ng/mL–10 μg/mL) [12].

For QC samples prepared by spiking human plasma with BPU, mmBPU, and aminoBPU, the within-run and between-run variability (precision), expressed as the percentage relative standard deviations, were less than 10.7%. The mean predicted concentration (accuracy) was less than 14.8% of the nominal value for the QC samples (Table 5). During in-study validation,

Table 5
Assessment of accuracy, precision, and recovery in plasma

Nominal concentration (ng/mL)	*n*	Accuracy (%)	Precision (%)		Recovery (%)
			Within-run	Between-run	
BPU plasma validation					
2.5	8	98.3	3.5	7.6	[a]
3.75	8	97.9	4.4	7.9	79.6
37.5	8	100.3	3.3	1.6	[a]
375	8	101.9	5.2	[b]	81.6
2500 (1:5)	12	103.2	10.0	[b]	[a]
2500 (1:10)	9	98.3	5.0	6.0	[a]
mmBPU plasma validation					
2.5	8	111.0	4.5	4.1	[a]
3.75	8	109.5	4.2	[b]	56.5
37.5	8	95.6	6.5	[b]	[a]
375	8	97.3	5.3	[b]	58.5
2500 (1:5)	12	102.0	10.7	[b]	[a]
2500 (1:10)	9	98.7	4.3	3.0	[a]
aminoBPU plasma validation					
2.5	8	114.8	1.0	3.0	[a]
3.75	8	104.5	4.1	4.2	47.8
37.5	8	99.3	3.9	[b]	[a]
375	8	99.8	4.7	[b]	50.2
2500 (1:5)	12	107.7	4.0	2.2	[a]
2500 (1:10)	9	102.7	3.5	6.4	[a]
BPU plasma in-study					
3.75	32	103.7	10.5	3.8	[a]
37.5	32	103.2	8.4	[b]	[a]
375	32	103.3	8.8	[b]	[a]
mmBPU plasma in-study					
3.75	32	109.3	12.8	3.1	[a]
37.5	32	103.6	8.4	[b]	[a]
375	32	103.6	8.9	[b]	[a]
aminoBPU plasma in-study					
3.75	32	107.9	7.5	6.7	[a]
37.5	32	103.2	6.2	3.4	[a]
375	32	103.2	6.2	[b]	[a]
BPU plasma cross-validation[c]					
5	15	103.6	8.3	7.7	[a]
150[d]	15	103.4	12.7	[b]	[a]
BPU plasma cross-validation[e]					
5	15	100.3	4.6	[b]	[a]
150[f]	15	103.2	3.4	1.9	[a]

[a] ND, not done.
[b] No significant additional variation was observed as a result of performing the assay in different runs.
[c] Cross-validation results using the original LC/MS/MS method.[12].
[d] Sample diluted 1:100 prior to analysis.
[e] Cross-validation results using the current LC/MS/MS method.
[f] Sample dilution was not necessary using the current LC/MS/MS method.

the within-run and between-run variability was less than 12.8% and accuracy was less that 9.3% of the nominal value. The relative recovery of BPU varied from 79.6% to 81.6% over the concentration range (Table 5). The relative recovery for mmBPU (56.5–58.5%) and aminoBPU (47.8–50.2%) were lower than BPU but consistent over the concentration range. In addition, a cross-validation was performed for 5 and 150 ng/mL between the original LC/MS/MS method and this method demonstrating nearly identical results (Table 5).

3.3.2. Urine

Prior analytical methods for BPU were for the plasma matrix [11,12]. To comprehensively study the clinical pharmacology of BPU, the expansion of the LC/MS/MS method to include other matrices was necessary. By utilizing a modified liquid–liquid extraction (*n*-butyl-chloride versus acetonitrile:*n*-butyl chloride (1:4, v/v)), appropriate sensitivity was achieved using LC/MS/MS for BPU, mmBPU, aminoBPU, G280, G308, and G322 [11].

Table 6
Assessment of accuracy, precision, and recovery in urine

Nominal concentration (ng/mL)	*n*	Accuracy (%)	Precision (%)		Recovery (%)
			Within-run	Between-run	
BPU urine validation					
0.1	6	105.0	8.7	[b]	[a]
0.15	6	111.1	0.0	3.47	98.1
0.7	6	96.4	10.6	[b]	[a]
18	6	104.8	6.5	[b]	97.6
mmBPU urine validation					
0.1	6	115.0	10.7	[b]	[a]
0.15	6	110.0	5.5	[b]	98.4
0.7	6	103.3	6.9	[b]	[a]
18	6	104.7	5.7	5.5	96.2
aminoBPU urine validation					
0.5	6	111.0	4.7	[b]	[a]
0.75	6	108.7	7.2	[b]	98.3
3.5	6	106.3	5.4	3.6	[a]
90	6	101.9	4.9	8.2	96.4
G280 urine validation					
10	6	103.3	2.5	17.7	[a]
15	6	98.8	11.1	[b]	77.5
70	6	102.5	3.4	4.0	[a]
1800	6	103.7	6.8	[b]	92.3
G308 urine validation					
1	6	96.7	24.7	[b]	[a]
1.5	6	95.6	17.5	[b]	80.3
7	6	104.5	16.3	4.6	[a]
180	6	102.7	4.8	[b]	84.4
G322 urine validation					
3	6	91.2	5.9	2.1	[a]
4.5	6	108.5	4.2	[b]	79.6
21	6	102.9	8.9	9.3	[a]
540	6	102.9	6.0	1.3	88.4
BPU urine in-study					
0.15	8	111.8	6.9	[b]	[a]
0.7	8	108.1	4.7	4.5	[a]
18	8	102.8	5.2	5.8	[a]
mmBPU urine in-study					
0.15	7	111.2	6.7	8.9	[a]
0.7	8	106.0	5.1	1.2	[a]
18	8	104.2	3.7	7.1	[a]
aminoBPU urine in-study					
0.75	8	101.4	6.9	3.1	[a]
3.5	8	104.1	4.2	4.5	[a]
90	8	100.0	2.4	6.5	[a]
G280 urine in-study					
15	8	101.9	8.0	2.7	[a]
70	8	97.3	6.2	7.5	[a]
1800	8	98.1	5.4	5.4	[a]
G308 urine in-study					
1.5	8	103.0	3.8	4.7	[a]
7	8	110.9	5.7	[b]	[a]
180	8	99.9	2.3	9.3	[a]
G322 urine in-study					
4.5	8	102.2	2.4	5.6	[a]
21	8	101.1	4.9	3.7	[a]
540	8	98.4	3.4	9.4	[a]

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

Table 7
Assessment of stability in plasma

Nominal concentration (ng/mL)	Stability (% of initial)				
	Freeze–thaw cycles[a]			Autosampler stability[b]	Long-term stability (−70 °C)[c]
	1	2	3		
BPU					
3.75	97.2	96.0	95.2	93.1	116.4
375	102.7	99.1	96.8	103.7	100.5
mmBPU					
3.75	100.5	87.9	90.0	112.5	108.3
375	111.0	106.9	103.6	100.8	131.5
aminoBPU					
3.75	104.6	98.0	95.0	111.7	94.5
375	104.0	101.6	102.0	101.6	101.2

[a] Performed in triplicate.
[b] Performed repeatedly for 8.7 h with one sample.
[c] Performed at 138 days.

For QC samples prepared by spiking human urine with BPU, mmBPU, aminoBPU, G280, and G322, the within-run and between-run variability (precision), expressed as the percentage relative standard deviations, were less than 11.1%, except at the G280 LLOQ which was 17.7%. For G308, the accuracy and precision were within acceptable limits (<15%) for the calibrators (Table 4), as was the accuracy of the QC samples but the precision was not (>15%) (Table 6). Therefore, G308 will only be used for qualitative analysis. During in-study validation, the within-run and between-run variability were less than 9.4% and accuracy were less that 11.8% of the nominal value (Table 6). The relative recovery of BPU (97.6–98.1%), mmBPU (96.2–98.4%), and aminoBPU (96.4–98.3%) from urine is presented in Table 6. The relative recovery for G280 (77.5–92.3%), G308 (80.3–84.4%), and G322 (79.6–88.4%) were lower than BPU, mmBPU, and aminoBPU and displayed more variability over the two concentrations tested.

3.4. Analyte stability

3.4.1. Plasma

BPU, mmBPU, and aminoBPU were stable on the auto-injector at room temperature for 9 h or up to approximately 90 injections when protected from light with aluminum foil covering the autosampler (Table 7). BPU, mmBPU, and aminoBPU were stable after three freeze–thaw cycles at −70 °C (Table 7). Long-term stability studies from 138 days demonstrate aminoBPU is stable but there is a slight increase in BPU at the lower concentration (116.4% at 3.75 ng/mL) and mmBPU at the higher concentration (131.5% at 375 ng/mL) (Table 7).

3.4.2. Urine

BPU, mmBPU, aminoBPU, G280, G308, and G322 were stable on the auto-injector at room temperature for 20.2 h or up to approximately 200 injections when protected from light with aluminum foil covering the autosampler (Table 8). In addition, urine containing BPU, mmBPU, aminoBPU, G280, and G322 was stable at room temperature for up to 5 h, as demonstrated by less than 12.1% deviation in concentration over these 5 h (Table 8). Initially, G308 is stable at room temperature for 1 h as the concentration decreased by 11.7% (data not shown) but by 5 h, the concentration falls to 16.4%. Freeze–thaw stability was not assessed since multiple aliquots were available for quantitation of patient samples. Long-term stability studies from 91 days demonstrate that both aminoBPU and G322 are stable with a slight increase in G308 high concentrations (117.6% at 180 ng/mL) and a decrease in BPU (83.4% at 0.15 ng/mL), mmBPU (68.9% at 18 ng/mL), and G280 (83.7% at 15 ng/mL) concentrations (Table 8).

3.5. Plasma concentration–time profile

3.5.1. Plasma

This LC/MS/MS method was applied to the quantitation of BPU and metabolites in plasma samples from a patient who has received BPU at a dose of 150 mg [9]. A single patient concentration versus time profile is illustrated in Fig. 4 after a single dose of

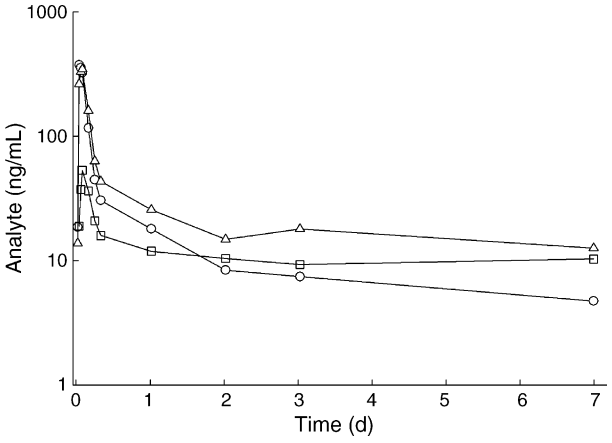

Fig. 4. Plasma concentration time curve in a single patient for BPU administered orally at a dose of 150 mg. The open circle (◯) represents BPU concentrations, the open triangle (△) represents mmBPU concentrations, and the open square (□) represents aminoBPU concentrations.

Table 8
Assessment of stability in urine

Nominal concentration (ng/mL)	Stability (% of initial)		
	Short-term (room temperature)[a]	Autosampler stability (room temperature)[b]	Long-term stability (−70 °C)[c]
BPU urine			
0.15	103.9	99.1	83.4
18	105.3	93.2	87.5
mmBPU urine			
0.15	96.0	93.5	95.7
18	100.9	89.9	68.9
aminoBPU urine			
0.75	103.7	87.0	90.2
90	101.6	91.4	106.5
G280 urine			
15	112.1	101.4	83.7
1800	110.5	112.9	93.1
G308 urine			
1.5	83.6	97.0	108.6
180	94.0	104.6	117.6
G322 urine			
4.5	90.8	96.5	99.1
540	100.6	107.6	109.4

[a] Performed at room temperature at 5 h with $n = 3$ sample.
[b] Performed repeatedly for 20.2 h with one sample.
[c] Performed at 91 days.

oral BPU. This patient had a maximum concentration (C_{max}) of 374.0 ng/mL for BPU, 345.0 ng/mL for mmBPU, 53.1 ng/mL for aminoBPU that occurred at 1.0, 2.0, and 2.0 h, respectively. The half-life and apparent oral clearance were 145.4 h and 41.6 L/h for BPU, 349.7 h and 14.3 L/h for mmBPU, longer sampling is necessary to determine the half-life for aminoBPU.

3.5.2. Urine

This LC/MS/MS method was applied to the quantitation of BPU and metabolites in urine samples from a patient who has received BPU at a dose of 150 mg [9]. As mentioned in Section 3.3.2, G308 was not quantitated due to poor assay performance. In the first 24 h after drug administration, 0.00049% of BPU parent compound was excreted as unchanged drug in the urine. As for the metabolites, 0.00065% of mmBPU, 0.0038% of aminoBPU, 0.35% of G280, and 0.037% of G322 were excreted in the first 24 h. In the first 72 h after drug administration, 0.00060% of BPU, 0.00086% of mmBPU, 0.0086% of aminoBPU, 0.61% of G280, and 0.084% of G322 were excreted in the first 72 h. Qualitatively, G308 was excreted in the urine over the first 72 h following drug administration (Fig. 5).

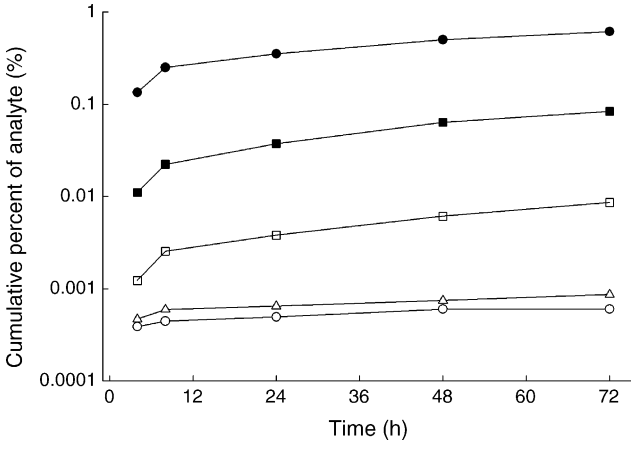

Fig. 5. Cumulative percent of BPU, mmBPU, aminoBPU, G280, and G322 excreted in the urine over 72 h for BPU administered orally at a dose of 150 mg. The open circle (○) represents BPU concentrations, the open triangle (△) represents mmBPU concentrations, the open square (□) represents aminoBPU concentrations, the closed circle (●) represents G280, and the closed square (■) represents G322.

4. Conclusions

In conclusion, we have developed and validated a LC/MS/MS assay for measuring BPU and its metabolites (mmBPU, aminoBPU, G280, and G322) concentrations in human plasma or urine under current requirements as to validation of bioanalytical methodologies [14]. The described method permits the analysis of patient plasma samples to concentrations of 2.5–500 ng/mL for BPU, mmBPU, and aminoBPU, which is sufficiently sensitive to allow pharmacokinetic monitoring after oral administration of BPU at doses of 150 mg. The described method permits the analysis of patient urine samples to concentrations of 0.1–20 ng/mL for BPU, 0.1–20 ng/mL mmBPU, 0.5–100 ng/mL aminoBPU, 10–2000 ng/mL G280, and 3–600 ng/mL G322 which is sufficiently sensitive to allow pharmacokinetic monitoring after oral administration of BPU at doses of 150 mg. Since G308 did not meet standard acceptance criteria, this was monitored qualitatively in urine. This LC/MS/MS method will

be used to measure BPU and metabolite concentrations in human plasma and urine to fully characterize the clinical pharmacology of this agent.

Acknowledgments

This work was supported by National Institutes of Health grants P30CA069773 and the Commonwealth Foundation for Cancer Research. We would like to thank Susan Davidson for quality assurance of the data contained in this manuscript.

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