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Journal of Chromatography B, 704 (1997) 277–288

JOURNAL OF CHROMATOGRAPHY B

Stability-indicating high-performance liquid chromatographic assay of busulfan in aqueous and plasma samples

Diana S.-L. Chow[a,*], Harshal P. Bhagwatwar[a], Sasima Phadungpojna[a], Borje S. Andersson[a,b]

[a]*Department of Pharmacological and Pharmaceutical Sciences, College of Pharmacy, University of Houston, Texas Medical Center, 1441 Moursund Street, Houston, TX 77030, USA*

[b]*Department of Hematology, The University of Texas, M.D. Anderson Cancer Center, Houston, TX 77030, USA*

Received 1 August 1996; received in revised form 30 July 1997; accepted 21 August 1997

Abstract

A sensitive, specific and stability-indicating high-performance liquid chromatographic (HPLC) assay, involving pre-column derivatization and solid-phase extraction (SPE), was developed and validated for the quantitation of busulfan (BU) in aqueous and plasma samples. The linearity of the assay was in the concentration ranges of 0.15–10 μg/ml and 0.15–3 μg/ml for aqueous and plasma samples, respectively. The within-day and between-day variations were 2.90 and 3.31%, respectively, for the aqueous samples, and 9.24 and 14.56%, respectively, for the plasma samples. The overall recovery, derivatization yield and SPE efficiency of BU from plasma samples were 82.03, 108.01 and 86.69%, respectively. Forced degraded samples, either in highly acidic, neutral or basic medium, produced no interfering peaks in the chromatogram. The reported assay requires only 0.2 ml of plasma for the analysis, and its sensitivity is 150 ng/ml by monitoring samples at a wavelength of 254 nm, sufficient to study the plasma pharmacokinetics of BU in rats after a clinically relevant oral dose. Moreover, the sensitivity of the assay can be significantly increased to 30 ng/ml by monitoring samples at a wavelength of 278 nm. The applications of the assay were demonstrated with BU solubility measurements in two aqueous systems and with plasma samples from a Sprague–Dawley rat for an in vivo pharmacokinetic study. In addition, the assay has been employed in the development of a patented intravenous formulation, and in evaluations of stability, preclinical pharmacokinetics in rats and dogs, and clinical phase I trial of the formulation. The assay is readily adaptable to clinical therapeutic drug monitoring.

Keywords: Busulfan

1. Introduction

Busulfan (BU, 1,4-butanediol dimethanesulfonate, with the structure shown in Fig. 1A, Myleran), a bifunctional alkylating agent, has been used since the 1950s for the palliative treatment of chronic myelogenous leukemia (CML) and other myeloproliferative syndromes [1]. In the past decade, high dose oral BU (1 mg/kg body weight every 6 h for 4 days) was combined with cyclophosphamide (CY) [2–4] as a preparative regimen for bone marrow transplantation (BMT). The BUCY conditioning regimen has become an important alternative to total body irradiation (TBI) based regimen prior to BMT.

*Corresponding author.

0378-4347/97/$17.00
PII S0378-4347(97)00419-2

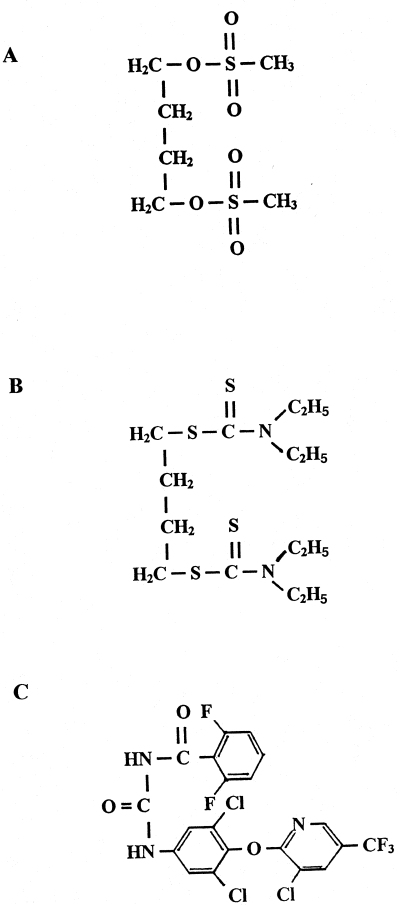

Fig. 1. Chemical structures of (A) busulfan, 1,4-butanediol dimethanesulfonate, (B) DDCB, 1,4-bis(diethyldithiocarbamoyl butane) and (C) internal standard CGA-112913, N-(2,6-difluorobenzoyl) - N′ - [3, 5-dichloro-4-(3-chloro-5-trifluoromethyl-pyridin-2-yloxy)-phenyl]-urea.

Clinically, oral BU causes severe mucositis, gastrointestinal irritation and neurotoxicity [2,5]. Nausea and vomiting are common, and result in imprecise dose due to the various degrees of drug loss in the vomit. As a result, the bioavailability of oral BU is highly variable.

The major dose-limiting toxicity of high-dose BUCY is hepatic veno-occlusive disease (HVOD). A significant correlation of plasma concentrations and systemic exposure of BU with HVOD was established in adults [6]. As a consequence, the importance of therapeutic drug monitoring and dose adjustment of BU during the course of high-dose regimen began to be accentuated in the past few years [7–9]. However, to routinely conduct BU monitoring and dose modification, one pre-requisite is to have a sensitive, specific and reproducible assay.

Several gas chromatographic (GC) assays for BU in plasma or aqueous solutions have been developed: GC with electron capture detection (ECD) [10–12], and GC–mass spectrometry (MS) with selected-ion monitoring (SIM) [13,14]. These assays offer high sensitivity and have been used in clinical disposition and pharmacokinetics studies [15,16]. However, in view of the availability of the analytical equipment, GC assays may not be applicable for routine drug monitoring in most of clinical settings.

Pichini et al. [17] developed an HPLC–MS (SIM) assay for BU in human serum and cerebrospinal fluid; nevertheless, the assay still involved the expensive mass spectrometer. The BU molecule contains no chromophore and absorbs no ultraviolet or visible (UV–Vis) light. Thus, a derivatization adding a chromophore to BU is essential for UV detection.

Blanz et al. [18] developed an HPLC assay which involved pre-column derivatization with iodide leading to 1,4-diiodobutane, followed by a post-column photolysis with the formation of iodide ions for UV detection. Henner et al. [19] and MacKichan and Bechtel [20] reported pre-column derivatization HPLC assays for BU in plasma. Most recently, Funakoshi et al. [21] developed an HPLC assay involving on-line derivatization and column switching for BU determination in human serum. In their methods [19–21], BU was derivatized with diethyldithiocarbamate (DDTC) to yield 1,4-bis-(diethyldithio-carbamoyl) butane (DDCB, Fig. 1B). These procedures offer simple and convenient alternatives to the GC assays. However, their applications in pharmacokinetic studies were not as well established as the available GC methods, and none of the above HPLC assays were documented as stability-indicating.

In our laboratory, an i.v. formulation of BU was developed [22,23]. In the process of the product development, a sensitive HPLC assay for the quantitation of BU in both aqueous and plasma samples was developed. The assay was employed to evaluate the formulation stability and preclinical pharmacokinetics of parenteral BU in rats [24] and dogs [25]. Currently, the assay is adopted for clinical

pharmacokinetic studies in patients [26,27]. This assay is adaptable to routine BU monitoring in a clinical setting.

This paper first describes the assay for BU in aqueous solutions. Its application in determining BU solubilities in two liquid formulations is demonstrated. Then, the BU assay for plasma samples which was modified with respect to the extraction and derivatization procedures from the assay of MacKichan and Bechtel [20] is presented. The extraction efficiencies for BU and the internal standard from each of the three key steps of the plasma assay are reported. The application of the assay in a pharmacokinetic study is demonstrated.

2. Experimental

2.1. Materials

The internal standard, N-(2,6-difluorobenzoyl)-N′-[3,5-dichloro-4-(3-chloro-5-trifluoro-methyl-pyridin-2-yloxy)phenyl]-urea (CGA-112913, Fig. 1C), was a gift from Ciba-Geigy (Basel, Switzerland). Hydroxypropyl betacyclodextrin (HBCD) was a gift from Pharmatec (Alachua, FL, USA). BU and DDTC were purchased from Sigma (St. Louis, MO, USA). The DDTC was stored in a desiccator at −20°C. Acetonitrile, tetrahydrofuran, methanol and ethyl acetate were all HPLC grade and purchased from VWR Scientific (Houston, TX, USA). N,N-Dimethylacetamide (DMA) was purchased from Aldrich (Milwaukee, WI, USA). Distilled deionized water was generated using Corning Mega-Pure apparatus (Corning, NY, USA) for preparations of HPLC mobile phase and sample solutions. Phenytoin (Dilantin capsule, 100 mg; Park-Davis, Morris Plains, NJ, USA), phenobarbital (sodium phenobarbital, USP grade, American Drug and Chemical, Los Angeles, CA, USA), pentobarbital (Nembutal Sodium solution, Abbott Labs., North Chicago, IL, USA), lorazepam (Ativan injection, 2 mg/ml; Wyeth-Ayerst, Philadelphia, PA, USA), meclizine (Antivert tablet, 12.5 mg; Roerig, New York, NY, USA), and acyclovir (Recordati Industria Chimica E Farmaceutica, Milan, Italy) were used to verify the assay specificity for clinical application.

2.2. Methods

2.2.1. Preparation of solutions

Stock solutions of the internal standard, CGA-112913 (200 and 40 μg/ml in methanol, respectively) were prepared and stored at −20°C until use. A solution of BU (1 mg/ml in DMA) was freshly prepared daily and used to prepare all the calibration standards. A solution of DDTC (1.17 *M* in water) was freshly prepared daily and kept at 4°C between uses within the day.

2.2.2. Derivatization and calibration curves of BU samples

2.2.2.1. *Authentic DDCB preparation and its stability in mobile phase*. BU was reacted with DDTC to yield DDCB using the procedure of Henner et al. [19]. The authentic DDCB was stored at −20°C until it was used to determine the extraction efficiency and overall recovery of BU. The structure of DDCB was confirmed with nuclear magnetic resonance (NMR) (data not shown).

To evaluate the stability of the reconstituted DDCB at room temperature, the authentic DDCB (5 μg/ml) was dissolved in the mobile phase of the HPLC assay. After the addition of internal standard, the solution was injected into the HPLC and the peak height ratio was monitored at various time points for a period of three days.

2.2.2.2. *Aqueous samples*. A 40-μl aliquot of the BU solution in DMA was mixed with water (3.96 ml) to give an aqueous BU solution of 10 μg/ml. Various volumes of this solution (0.1–2.0 ml) were mixed with distilled deionized water to yield 2-ml working solutions of 0.15–10 μg/ml. Samples (0.5 ml) of the aqueous BU solutions were mixed with 20 μl of the CGA-112913 solution (200 μg/ml) in a 5-ml disposable borosilicate glass culture tube (Kimax, VWR Scientific, Houston, TX, USA), to which 0.5 ml of the DDTC solution was added. The mixture was vortexed for 30 s, and then rotated on a Tube Rotator (Scientific Equipment Products, Baltimore, MD, USA) for 5 min. The DDCB was extracted from the reaction mixture with 2 ml of ethyl acetate by vortexing for 1 min and then centrifugation for 10 min at 5125 *g* (International

Clinical Centrifuge, Model CL, International Equipment, Needham, MA, USA). A 1-ml volume of the ethyl acetate layer was withdrawn and evaporated to dryness under compressed air with a Meyer N-Evap Analytical Evaporator (Organomation Associates, Northborough, MA, USA). The residue was reconstituted by vortexing for 10 s with 1 ml of the mobile phase of the HPLC assay.

2.2.2.3. *Plasma samples*. A 0.1-ml aliquot of the BU solution in DMA was mixed with 4.9 ml of DMA to produce a BU solution of 20 μg/ml. This solution was diluted with various volumes of DMA (0–0.95 ml) to make BU/DMA solutions in a concentration range of 1–20 μg/ml. Pooled rat plasma (0.2 ml) was spiked with 30 μl of these solutions to yield final BU concentrations of 0.15–3 μg/ml. A 20-μl volume of the diluted CGA-112913 solution (40 μg/ml) was vortexed with each of the mixtures. The plasma proteins were precipitated with 0.2 ml acetonitrile and then vortexed for 30 s. The mixtures were then centrifuged at 16 750 *g* (HBI microcentrifuge, Fisons, Oxbridge, UK) for 3 min and 0.36 ml of the supernatant was transferred into a disposable borosilicate glass culture tube containing 0.4 ml of water. BU was derivatized with 0.2 ml of DDTC solution. The mixing and extraction procedures were the same as those previously described for aqueous samples. The samples were centrifuged for 10 min at 5125 *g* (International Clinical Centrifuge, Model CL) and 1.8 ml of the ethyl acetate layer was withdrawn and evaporated to dryness under compressed air at 45°C. The residue was reconstituted in 0.2 ml of methanol.

The DDCB and CGA-112913 were separated from the derivatization mixture and other plasma components using solid-phase extraction (SPE) with Sep-Pak cartridges (C_{18}, 1-ml capacity, syringe barrel, Millipore, Bedford, MA, USA). The cartridges were conditioned under unit gravity with seven 1-ml volumes of methanol followed by two 1-ml volumes of distilled deionized water. The residue dissolved in methanol was loaded on the cartridges to which 0.5 ml of water had been added and allowed to flow through under unit gravity. The cartridges were washed twice with 1 ml of 50% methanol in distilled deionized water (v/v). The DDCB and the CGA-112913 were then eluted from the columns under vacuum with 250 μl methanol twice, followed by two 0.5-ml volumes of ethyl acetate. The combined eluates were evaporated to dryness at 45°C with compressed air, and the residue was reconstituted with 0.2 ml of the mobile phase prior to HPLC analysis.

2.2.3. HPLC conditions

The liquid chromatograph used (Consta-Metric I, LDC Analytical, Riviera Beach, FL, USA) was equipped with a 100-μl sample loop (Valco Instruments, Houston, TX, USA), a fixed-wavelength UV detector monitoring at 254 nm (UV-III monitor, LDC Analytical), a Microsorb-MV column (5 μm particle size, C_{18}, 25 cm×4.6 mm I.D., Rainin Instruments, Woburn, MA, USA), and a chart recorder (Linear Instrument, Irvine, CA, USA). Alternatively, a detector with variable-UV wavelengths (Spectromonitor-III, LDC Analytical) was used to monitor BU in stability-indicating study. The isocratic mobile phase was acetonitrile–tetrahydrofuran–distilled deionized water (11:4:5, v/v) (pH 4.2 without modification). The flow-rate was 1.2 ml/min and the recorder chart speed 20 cm/h.

2.2.4. Assay validation

Calibration curves for aqueous and plasma samples were constructed within the concentration ranges of 0.15–10 μg/ml and 0.15–3 μg/ml, respectively. Six sets of standard curves were prepared and analyzed on the same day to establish the within-day variation. The assay was repeated 6 and 15 times for aqueous and plasma samples, respectively, over a 14-month period with freshly prepared stock solutions to establish the between-day variation.

2.2.5. Solubility measurements

BU was equilibrated with solutions of 10 and 45% HBCD in water (w/w) for 2 h. All the solutions were filtered through a 0.45-μm Selas silver membrane filter (25 mm diameter, Nuclepore, Filtration Products, Pleasanton, CA, USA). The concentrations were measured after suitable dilution and derivatization.

2.2.6. Stability-indicating study

Aqueous solutions of BU (10 μg/ml) were mixed with equal volumes of water, 0.1 *M* NaOH (pH>

13), or concentrated HCl (pH<1.2) to yield BU concentrations of 5 μg/ml. After being vortexed for 1 min, these solutions were either kept at room temperature or boiled for 3 min in boiling water. Once they were cooled to room temperature, 20 μl of the CGA-112913 (200 μg/ml) was added to 0.5 ml of the above solutions. The solutions were derivatized and the DDCB and CGA-112913 were extracted as previously described. The residue was reconstituted with the mobile phase and injected into the HPLC, and monitored respectively at 230, 254, and 278 nm where DDCB absorbs significantly. The peak height ratios of DDCB/CGA-112913 at the three wavelengths were compared for unboiled and boiled solutions at the three pH conditions following the procedure of Lau et al. [28]. The chromatograms from force degraded solutions without CGA-112913 were also examined to verify the absence of interference.

2.2.7. Determination of extraction efficiencies

The three key steps of the procedure for plasma sample preparation were solvent extraction of BU from the plasma, derivatization of extracted BU and SPE of DDCB from the derivatization mixture. The extraction efficiencies of BU and CGA-112913 in each of the steps were determined by comparing the slope of the calibration curve derived from the analyte added before a particular step with that added after the step. The overall recovery was also evaluated.

2.2.8. Pharmacokinetic study

A male Sprague–Dawley rat (270 g, Sasco, Omaha, NE, USA) was cannulated at jugular vein with a soft silastic catheter (Silmed tubing, Products for Surgery, Houston, TX, USA) under pentobarbital sodium anesthesia (50 mg/kg body weight). After 24 h recovery, BU (0.5 mg/kg) was administered orally as a suspension in 0.4 ml of normal saline (BU from crushed Myleran tablets, 2 mg, Burroughs–Wellcome Pharmaceuticals, Research Triangle, NC, USA) via an oral gavage assembly, while the rat was under temporary Metofane anesthesia (Pitman-Moore, Mundelein, IL, USA). Blood samples (0.5 ml) were withdrawn via the jugular cannula at 5, 10, 20, 30 and 60 min, and 2 and 6 h post dose and centrifuged at 13 500 *g* (Eppendorf Centrifuge 5415, Brinkmann Instruments, Westbury, NY, USA). The plasma was collected and frozen at −20°C until analyzed. The withdrawn blood volume was replaced with an equal volume of heparinized saline.

2.2.9. Assay specificity for clinical drug monitoring

The assay specificity was imperative to establish for the potential clinical application of the assay, because patients receiving BU are commonly under concomitant medications to manage the disease state, or to prevent/alleviate the adverse effects of BU treatment. These drugs include, but not limited to, anticonvulsant, antiemetic and antiviral agents.

To establish the specificity, six agents, namely, phenytoin, phenobarbital, pentobarbital, lorazepam, meclizine, and acyclovir were individually dissolved in a suitable solvent (water or DMA) and spiked in a blank plasma sample at a concentration range of 0.1–1.0 mg/ml. The potential interference of the agents were examined chromatographically following the above described assay procedures. These six agents were also dissolved in the mobile phase of the HPLC assay and injected directly to determine their individual retention times.

In addition, a plasma sample from a patient (AS), who received eight medications (Dilantin, Pen VK, Norfloxacin, Bactrim, Carafate, Valacyclovir, Vitamin K and Zofran) for two days prior to BU infusion, was also examined before the treatment to demonstrate the specificity of the BU assay by establishing the lack of interference from these medications.

3. Results and discussion

3.1. HPLC assay optimization

Baseline resolution of DDCB and CGA-112913 was achieved with retention times of 7.5 and 9 min, respectively, for both aqueous and plasma samples (Figs. 2 and 3). DDTC produced no interference to the two analytes in the chromatograms. Addition of acetonitrile to the mobile phase yielded a sharper DDCB peak, when compared with that from the methanolic mobile phase used by Henner et al. [19]. Initially, butylated hydroxytoluene (BHT) was tried as an internal standard, but it was unstable in

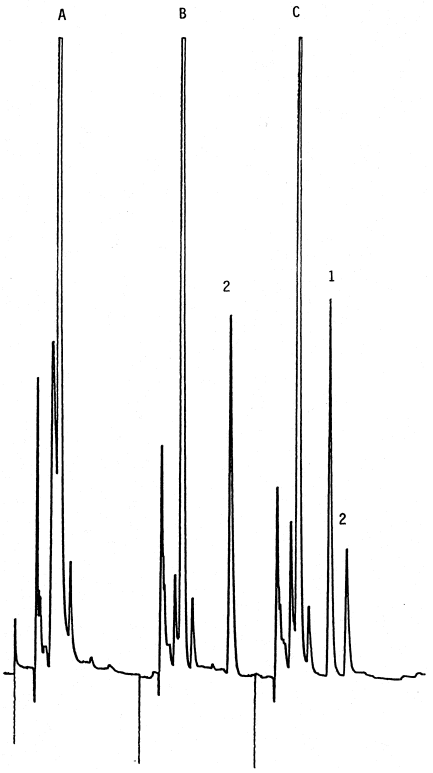

Fig. 2. Authentic HPLC chromatograms from the aqueous assay. (A) Blank derivatized water, (B) blank with CGA-112913, (C) aqueous sample. Peaks: 1=DDCB; 2=CGA-112913. All monitored at 254 nm and 0.016 a.u.f.s.

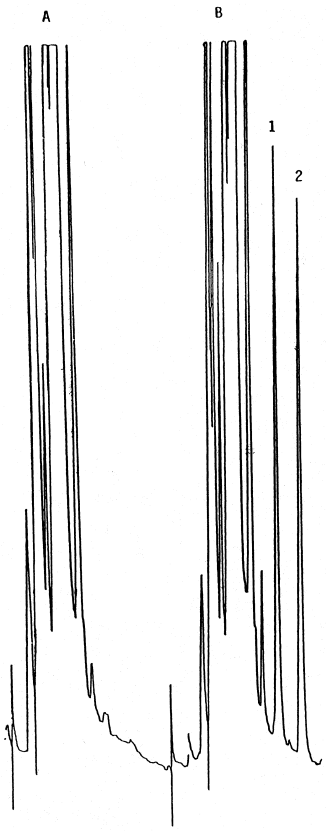

Fig. 3. Authentic HPLC chromatograms from the plasma assay. (A) Blank derivatized plasma, (B) plasma sample. Peaks: 1= DDCB; 2=CGA-112913. All monitored at 254 nm and 0.008 a.u.f.s.

solution and interacted with DDTC, interfering with the derivatization reaction of BU. To avoid this problem, BHT had to be added at the very end before reconstitution, that defeated the purpose of using an internal standard. In contrast, CGA-112913 did not react with DDTC, inasmuch as the peak heights, shapes and retention times of CGA-112913 were identical before and after derivatization with DDTC.

Denatured ethanol was first used to prepare the stock solutions of BU with a solubility of 1 mg/ml for the standards. Sonication was used to facilitate the solubilization process because the dissolution rate of BU in ethanol is slow. However, the results using these standard solutions were irreproducible. We therefore prepared the stock solutions in DMA, in which BU has a solubility in excess of 75 mg/ml and dissolves instantaneously.

Kazemifard and Morgan [29] could not reproduce the chromatogram by the procedure of Henner et al. [19] and reported that even with a prolonged incubation of BU with DDTC, the derivatization was incomplete and interfering peaks developed in the chromatogram. The result of our initial trial concurred with their observations. To optimize the derivatization condition, the DDTC concentration was varied from 0.3 to 1.75 *M* and the reaction time from 30 s to 120 min (data not shown). The optimal condition was selected with DDTC concentration of 1.17 *M* and the reaction time of 5 min. Decreasing the pH from 10 (pH of a mixture of DDTC and BU) to 7 using either ammonium acetate or a phosphate buffer did not affect the derivatization yield.

The peak homogeneity of DDCB and CGA-112913 was verified by employing different mobile phases. Lowering of the acetonitrile content of the mobile phase from 55 to 50% (v/v) while keeping

the tetrahydrofuran content constant did not further resolve additional peaks from the existing ones. Similarly, increasing tetrahydrofuran concentration from 20 to 25% (v/v) did not produce any additional peaks in the chromatogram. The peaks of DDCB and CGA-112913 contained no co-eluting compounds.

3.2. Aqueous samples

SPE was unnecessary for aqueous samples to yield a chromatogram of good resolution without any interference (Fig. 2). The aqueous assay was linear within the concentration range of 0.15–10 μg/ml. The assay was reproducible with within-day and between-day variations of 2.90 and 3.31%, respectively (Table 1). The lower limit of detection was 0.125 μg/ml at a signal-to-noise ratio of 4. The DDCB was stable in the mobile phase for three days, and so an autosample injector can be used for routine HPLC analysis.

Ethyl acetate was found to be a good extracting solvent for DDCB and CGA-112913. CGA-112913 was recovered quantitatively, as evidenced by the ratio of the standard curves prepared with CGA-112913 added before derivatization to that after reconstitution. In addition, no change in the absolute peak height of the DDCB or CGA-112913 was observed, indicating that CGA-112913 did not interfere with the derivatization reaction.

3.3. Solubility measurement

The developed aqueous assay was applied to measure the solubilities of BU in two aqueous cyclodextrin systems. The solvent systems tested increased the aqueous BU solubility 9- and 27-fold, respectively (Table 2), as compared with its solubility in water, 0.1 mg/ml.

Table 2
Solubility of BU in various solvent systems

Formulation	Concentration (mg/ml)
HBCD 10% (w/w)	0.923 (0.08)
HBCD 45% (w/w)	2.703 (0.07)

(n=3), numbers in parentheses indicate standard deviations.

3.4. Stability-indicating capability

The degradation products in both acidic and alkaline conditions did not interfere with the quantitation of BU.

The BU peak homogeneity from force degraded samples at neutral and alkaline conditions was verified. The change of peak-height ratio of DDCB/CGA-112913 at different wavelengths with and without boiling was less than 12% under all conditions (Table 3). The chromatograms showed no interfering peaks at the retention times of DDCB or CGA-112913 for these degraded solutions. Thus, the presence of co-eluting compounds with DDCB and CGA-112913 was very unlikely (Fig. 4).

Further proof of peak homogeneity was obtained by establishing three calibration curves in the BU concentration range of 0.15–10 μg/ml at wavelengths of 230, 254 and 278 nm, respectively. These calibration curves were used independently to determine the concentrations of BU in the degraded solutions (pH>13). The three concentrations thus

Table 1
Calibration curves of BU for aqueous and plasma samples

Parameter	Aqueous samples	Plasma samples
Concentration range (μg/ml)	0.15–10	0.15–3
Slope	0.230 (0.008)	0.463 (0.067)
Within-day variation (%)	2.90	9.24
Between-day variation (%)	3.31	14.56
Intercept	−0.121 (0.30)	0.0098 (0.034)
Correlation coefficient	0.9933	0.9930
Limit of detection[a] (μg/ml)	0.125	0.15
n	6	15

[a] At signal-to-noise ratio=4.
Numbers in parentheses are standard deviations.

Table 3
Comparison of peak height ratios of DDCB/CGA-112913 at different wavelengths

Condition	Sample	PHR_{230}/PHR_{254}	PHR_{230}/PHR_{278}	PHR_{254}/PHR_{278}
Water	Unboiled	0.6466	0.3105	0.4801
	Boiled	0.6537	0.3519	0.5384
	% Change	1.09	11.77	10.83
NaOH	Unboiled	0.6638	0.3197	0.4816
	Boiled	0.7471	0.3256	0.4358
	% Change	11.15	1.81	10.51

PHR_{230}, PHR_{254} and PHR_{278} are peak height ratio recorded at 230, 254 and 278 nm, respectively.
BU degrades within seconds in concentrated HCl even without boiling, so it was impossible to obtain its PHR with CGA-112913.

obtained were in close agreement with each other (Table 4).

Under acidic conditions (pH<1.2), all the BU was hydrolyzed regardless whether the solution was boiled or not. However, the chromatogram indicated that the degradation products were very hydrophillic and/or were not derivatized by DDTC. Tetrahydrofuran and methanesulfonic acid, which are known degradation products of BU [30,31], were eluted with the solvent front, and no additional peaks were observed.

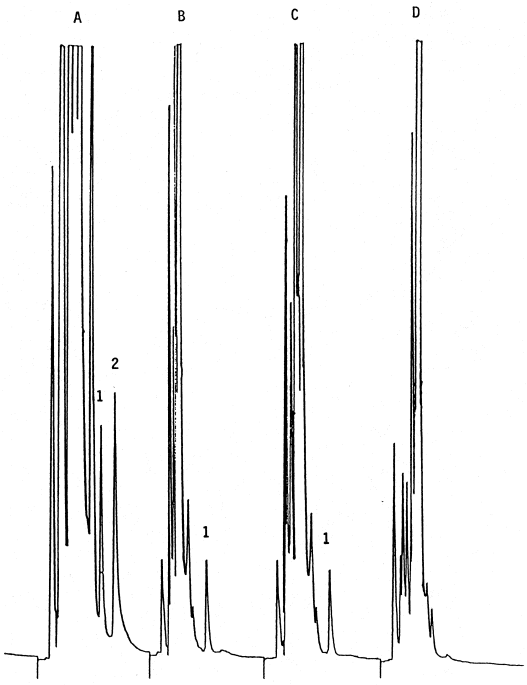

Fig. 4. Chromatograms of BU in (A) undegraded aqueous solution, 1=DDCB, 2=CGA-112913; (B) aqueous solution after boiling; (C) solution in 0.1 *M* NaOH after boiling; (D) solution in concentrated HCl after boiling. All solutions were monitored at 254 nm and 0.016 a.u.f.s. Solutions for chromatograms B, C and D did not contain CGA-112913.

3.5. Plasma samples

The plasma assay was linear within the concentration range of 0.15–3 μg/ml plasma. The assay was reproducible with within-day and between-day variations of 9.24 and 14.56%, respectively. The lower limit of detection was 0.15 μg/ml (Table 1). Importantly, monitoring at 278 nm instead of 230 and 254 nm significantly increased the sensitivity of the assay to 30 ng/ml (detailed data not shown), based on the slopes of the calibration curves established at various wavelengths (Table 4).

To precipitate plasma proteins, acetonitrile was a better reagent than methanol and yielded a cleaner solvent front in the chromatogram that did not interfere with the analyte peaks. With methanol, the solvent front completely obscured the analyte peaks. For the extraction of BU from plasma samples, chloroform was initially used but it brought in interference to the chromatogram. Ethyl acetate was selected because it had high extraction efficiency for the two analytes and yielded an interference-free chromatogram.

The SPE of DDCB was essential for plasma

Table 4
Peak homogeneity of the BU HPLC assay

Parameter	Wavelength (nm)		
	230	254	278
Calibration curves			
Slope	0.109	0.172	0.351
Intercept	−0.002	−0.014	−0.029
Correlation coefficient	0.999	0.998	0.994
BU remaining after degradation in 0.1 *M* NaOH[a] (μg/ml)	1.469 (0.154)	1.314 (0.050)	1.468 (0.062)

[a] Initial concentrations of the solutions were 5 μg/ml, boiled for 3 min.
Numbers in parentheses indicate standard deviations of three determinations.

samples. Without the SPE, a large solvent front obscured the two analyte peaks. The combined methanolic and ethyl acetate extracts were evaporated under compressed air at 45°C in a water bath. The stability of the DDCB or CGA-112913 was unaffected at this temperature. This was confirmed in a separate experiment where a solution of DDCB and CGA-112913 was prepared in a mixture of methanol (0.5 ml) and ethyl acetate (1 ml), and then evaporated to dryness in the condition mentioned above. The peak height ratio from the sample was compared with that in which the DDCB and CGA-112913 were dissolved in the mobile phase and injected directly into the HPLC without any extraction. There was no significant difference between the two samples.

3.6. Extraction efficiency

The extraction recoveries of BU and CGA-112913 from plasma were 90.68% and 89.95%, respectively. The derivatization yield of DDCB was 108.01%. The SPE recovered 89.69% and 101.45% of DDCB and CGA-112913, respectively. The overall recovery for BU and CGA-112913 were 82.03 and 91.24%, respectively (Table 5).

Table 5
Extraction efficiencies of BU and CGA-112913 from plasma samples

No.	Procedure	DDCB		CGA-112913	
		Measurements[a]	Recovery[b] (%)	Measurements[a]	Recovery[b] (%)
1	Solvent extraction	(Plasma+BU)/ (BU after extraction)	90.68 (4.41)	(Plamsa+CGA-112913)/ (CGA-112913 after extraction)	89.5 (4.21)
2	Derivatization	(BU after extraction)/ DDCB after extraction)	108.01 (5.58)	N/A	N/A
3	Solid-phase extraction	(DDCB to Sep-Pak)/ (DDCB after reconstitution)	89.69 (2.14)	(CGA-112913 after extraction)/ (CGA-112913 after reconstitution)	101.45 (0.85)
4	Overall	(Plasma+BU)/ (DDCB after reconsitution)	82.03 (3.99)	(Plasma+CGA-112913)/ (CGA-112913 after reconstitution)	91.24 (4.27)

[a] Ratios of the slopes obtained from these two procedures.
[b] $n=3$, numbers in parentheses are standard deviations.
N/A=Not applicable, because internal standard was not reacted with the derivatizing agent.

The DDCB recovery from the Sep-Pak cartridges was lower than the 100% recovery reported by MacKichan and Bechtel [20]. However, MacKichan and Bechtel determined recovery by comparing the peak heights of a solution passed through the Sep-Paks once to that passed through twice. Such a comparison carried a risk of overestimating the recovery from the cartridges, because the potential adsorption of the analytes to the cartridges in the first elution might be overlooked. To avoid this possibility, we made a comparison between the peak height ratios of a DDCB solution before and after passing through the cartridge. In addition, we determined the recoveries by using calibration curves instead of single-point determinations. Finally, we demonstrated that methanol alone was unable to elute the DDCB and CGA-112913 completely from the Sep-Paks and so we used ethyl acetate in subsequent elutions to achieve the recovery of 89.69%.

3.7. Pharmacokinetic study

The developed assay was applied to analyze plasma samples obtained after an oral BU dose (0.5 mg/kg) to a Sprague–Dawley rat. The pharmacokinetic profile is shown in Fig. 5. Clearly, the plasma assay was sensitive enough to detect and quantitate the BU plasma concentrations at least 6 h after a clinically relevant dose of the drug.

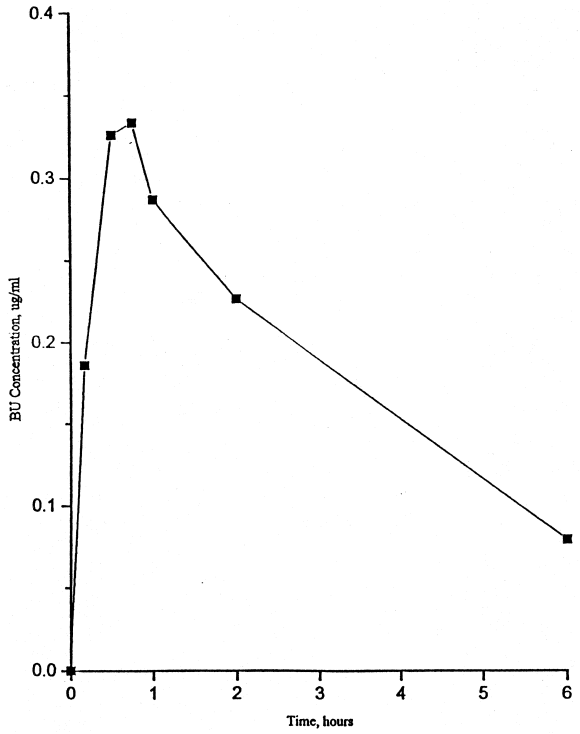

Fig. 5. Pharmacokinetic profile of BU administered orally to rats at a dose of 0.5 mg/kg body weight.

3.8. Assay specificity

The retention times of phenytoin, phenobarbital, pentobarbital, lorazepam, meclizine and acyclovir are 2.40, 2.35, 2.68, 2.63, 1.9 and 3.95 min, respectively, much shorter than those of DDCB and CGA-112913, 7.5 min and 9 min, respectively. When the agents were spiked in plasma samples, their peaks were obscured in the solvent front as expected, and demonstrated no interference for the two analytes. The typical chromatograms from the first three agents were shown in Fig. 6.

In addition, an interference-free chromatogram was also obtained from the patient AS (Fig. 7), indicating that the presence of these eight clinically concomitant medications in plasma did not interfere with the quantitation of BU.

4. Conclusions

A sensitive, specific and stability-indicating HPLC assay was developed and validated. We significantly improved the procedure of MacKichan and Bechtel [20] with respect to derivatization conditions and SPE. In addition, the authentic solid DDCB was used to determine the derivatization yield, SPE efficiency, and overall recovery of the sample preparation procedure.

Most of the published assays use 1–3 ml of human plasma for analysis, and are not verified for preclinical animal study with rats or mice where less than 0.3 ml is usually available for each plasma sample. The present work requires only 0.2 ml of plasma for the analysis, and the assay sensitivity is 150 ng/ml,

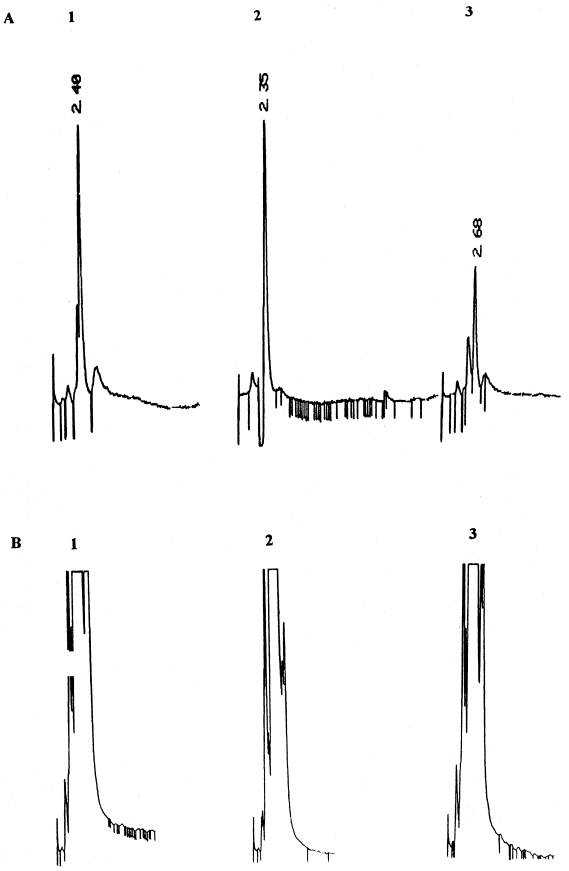

Fig. 6. Interference-free chromatograms from commonly concomitant medications of (1) phenytoin, (2) phenobarbital and (3) pentobarbital. (A) Retention behavior; (B) spiked plasma samples. All monitored at 254 nm and 0.008 a.u.f.s.

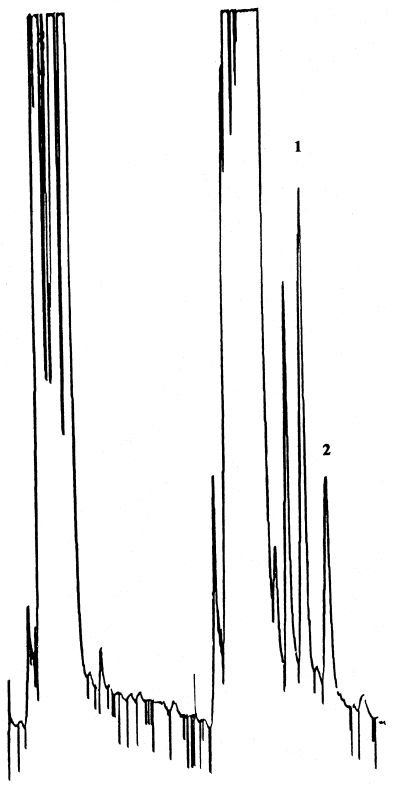

Fig. 7. Interference-free chromatograms from patient AS receiving eight other medications (Dilantin, Pen VK, Norfloxacin, Bactrim, Carafate, Valacyclovir, Vitamin K and Zofran) prior to BU infusion. (A) Plasma blank; (B) spiked plasma sample. Peaks: 1=DDCB; 2=CGA-112913. All monitored at 254 nm and 0.008 a.u.f.s.

sufficient to study the plasma pharmacokinetics of BU in rats after a single oral dose of 0.5 mg/kg body weight. Moreover, the sensitivity of the assay could be significantly increased to 30 ng/ml by monitoring samples at a wavelength of 278 nm.

This assay is applicable to aqueous samples for the development of other BU aqueous formulations, and the evaluation of BU stability in such formulations. In addition, the modified assay for plasma samples has been successfully employed in preclinical pharmacokinetic studies in rats [24] and dogs [25], and in clinical phase I and II trials [26,27], demonstrating that it is readily adaptable to therapeutic drug monitoring.

Acknowledgements

This project was made possible by a generous grant from the ADLER Foundation, New York, NY, USA.

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