Journal of Chromatography B, 917–918 (2013) 62–70

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Determination of tissue distribution of potent antitumor agent ureidomustin (BO-1055) by HPLC and its pharmacokinetic application in rats

Shin-I. Chien[a,b], Jiin-Cherng Yen[a], Rajesh Kakadiya[c], Ching-Huang Chen[c], Te-Chang Lee[c], Tsann-Long Su[c,**], Tung-Hu Tsai[d,e,*]

[a] Institute of Pharmacology, National Yang-Ming University, Taipei, Taiwan
[b] Department of Pharmacy, Far Eastern Memorial Hospital, Taipei, Taiwan
[c] Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan
[d] Institute of Traditional Medicine, National Yang-Ming University, Taipei, Taiwan
[e] Department of Education and Research, Taipei City Hospital, Taipei, Taiwan

ARTICLE INFO

Article history:
Received 23 May 2012
Accepted 29 December 2012
Available online 9 January 2013

Keywords:
Alkylating agent
Bioassay
Organ distribution
Pharmacokinetics

ABSTRACT

Ureidomustin hydrochloride (BO-1055) was designed as a water-soluble nitrogen-mustard, which exhibited potent anticancer activity and was selected as a candidate for preclinical studies. However, up to date, there is rarely an easy and economic method to quantize ureidomustin in the biological samples. The aim of this study is to develop a simple yet valid quantization method to tackle this challenge. Here we present a combined high-performance liquid chromatography with photodiode array (HPLC-PDA) method in quantizing the ureidomustin in the plasma and various organs of Sprague-Dawley rats. The method was validated in terms of precision, accuracy, and extraction recovery. Furthermore, the established method was applied to study pharmacokinetics of ureidomustin in the rat's plasma and verified via a liquid chromatography tandem mass spectrometry (LC–MS/MS) method. Calibration curves of the plasma and organ samples were falling at the range between 0.5–50 μg/mL and 0.1–50 μg/mL ($r^2 \geq 0.999$ and CV $\leq \pm 15\%$), respectively. The limits of detection (LOD) were 0.1 μg/mL for plasma samples and 0.05 μg/mL for organ samples, while the detection limits of quantification (LOQ) were 0.5 μg/mL for plasma samples and 0.1 μg/mL for organ samples. The average recovery of ureidomustin was about 83%. These results demonstrated a linear pharmacokinetic pattern at dosages of 10 and 30 mg/kg. The pharmacokinetic data revealed that ureidomustin was best fitted to a two-compartment model with a rapid distribution phase and a slow elimination phase. Besides, after a short intravenous administration time at the dose of 10 mg/kg, ureidomustin was found to be quickly distributed to all organs in rats, accumulated mainly in the kidney, and only a limited amount was detected in the brain.

1. Introduction

Among old drugs in chemotherapy regimens for cancer, nitrogen-mustard (N-mustard) is the oldest synthetic alkylating agent firstly reported for treating malignant diseases in 1942 [1]. N-mustard is one of the bi-function alkylating agent, which owes their cellular activity by producing intrastrand/interstrand DNA cross linking, or DNA-protein cross links [2]. Currently, N-mustard still plays an important role in treating cancers, including Hodgkin's disease, multiple myeloma, colon cancer, prostate cancer, etc. They are also used in combination with other drugs for the treatment of various tumors [3]. However, N-mustards, such as melphalan, mechlorethamine and cyclophosphamide are highly reactive agents. Aside from interacting with DNA, these agents are able to interact with other components in normal cells, producing undesirable side-effects. They also lack sequence-specific DNA binding and may induce carcinogenicity [4]. These agents may also lose their antitumor activity because the damaged DNA induced by N-mustard may be repaired. For these reasons, there has been considerable interest in developing new N-mustards that would be chemically stable and able to alkylate the DNA in a sequence-specific manner to reduce the unwanted side-effects [5]. To this end, numerous new N-mustards have been designed as prodrugs, consisting of chemically stable compounds with improved water-solubility and fewer side-effects [6–8].

Ureidomustin hydrochloride (BO-1055, Fig. 1) is a chemically stable and water-soluble DNA alkylating agent [9]. This agent has a phenyl N-mustard pharmacophore, which is linked to a benzamide

* Corresponding author at: Institute of Traditional Medicine, School of Medicine, National Yang-Ming University, 155, Li-Nong Street Section 2, Taipei 112, Taiwan. Tel.: +886 2 2826 7115; fax: +886 2 2822 5044.
** Co-corresponding author.
E-mail addresses: tlsu@ibms.sinica.edu.tw (T.-L. Su), thtsai@ym.edu.tw (T.-H. Tsai).

1570-0232/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jchromb.2012.12.025

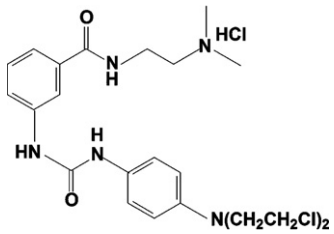

Fig. 1. Chemical structure of ureidomustin (BO-1055).

moiety via an ureido spacer. The benzamide moiety contains a water-soluble *N*,*N*-dimethylaminoethyl side-chain, which can form a water-soluble hydrochloride salt. The conjugate exhibits a broad spectrum of antitumor activity and has no cross-resistance to taxol or vinblastine. This agent also displays potent therapeutic efficacy against human breast cancer, and complete tumor remission was observed in a tumor xenograft model. Moreover, this agent exhibits potent antitumor activity against colon and prostate tumor xenografts, with more than 92% of the tumors suppressed. This derivative was thus selected as a candidate for preclinical studies.

As we know, the ureidomustin is the newer specific anticancer drug with limited preclinical data. Up to date, there is little information on how to assay the ureidomustin in the biological samples. Therefore, the aim of this study is to develop a simple, accurate, quick, sensitive and novel HPLC-PDA method for the analysis of ureidomustin in the biological samples and further to evaluate chemical properties and medicinal properties of for preclinical pharmacokinetic study. The LC–MS/MS was used to confirm that ureidomustin could be detected in the rat plasma. This method was also applied in the organ distribution of ureidomustin in the Sprague-Dawley rat.

2. Experimental

2.1. Chemicals and reagents

Ureidomustin, 1-[3-((2-(dimethylamino)ethyl)carbamoyl) phenyl]-3-[4-(bis(2-chloro-ethyl)amino)phenyl] urea hydrochloride (BO-1055) was synthesized according the procedure described previously [9]. Sodium chloride, heparin sodium and propyl paraben (internal standard) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Liquid chromatographic grade solvents and other reagents were purchased from E. Merck (Darmstadt, Germany). Pure water for all preparations was obtained by the Milli-Q system (Millipore, Bedford, MA, USA). The stock solution of ureidomustin (10 mg/mL in MeOH) was diluted with 50% acetonitrile to give a serial of working standard solutions. Propyl paraben (25 μg/mL) was dissolved in acetonitrile as internal standard solution.

2.2. HPLC-PDA instrumentation and method validation

The HPLC-PDA analysis of ureidomustin was firstly conducted by an HPLC system with a chromatographic pump (LC-20AT, Shimadzu, Kyoto, Japan) equipped with autosampler (SIL-20AT, Shimadzu), diode array detector (SPD-M20A, Shimadzu), and degasser (DG-2410). PDA was used to separate and quantize of ureidomustin by using a reversed-phase C18 column (4.6 mm × 150 mm, 5 μm, extend-C18, purchased from Agilent, Palo Alto, CA, USA) with a mobile phase consisting of acetonitrile–10 mM monosodium phosphate (28:72, v/v, pH 3.0 adjusted with orthophosphoric acid) being delivered isocratically at the flow rate of 1.0 mL/min. The UV wavelength was set at 275 nm, and the injection volume of all samples was 20 μL.The developed method was validated, according to the FDA guidelines [10]. Calibration curves were constructed by plotting the peak area ratio of ureidomustin and internal standard (ISTD) versus concentration. All calibration curves were required to have a coefficient correlation (r^2) value of at least 0.995. The calibration range at low, medium and high concentrations in five replications on the same day (intra-day) and five sequential days (inter-day) were prepared in the same manner to verify the precision and accuracy of the analyte for different biological matrix. The accuracy was calculated from the nominal concentration (C_{nom}) and the mean value of observed concentration (C_{obs}) as follows: accuracy (bias, %) = $[(C_{nom} - C_{obs})/C_{nom}] \times 100$. The precision (relative standard deviation, RSD) was calculated from the standard deviation and observed concentration as follows: precision (RSD, %) = [standard deviation (SD)/C_{obs}] × 100. The percentage of bias (% bias) and the percentage of RSD (% RSD) for the lowest acceptable reproducibility concentration were defined as being within ±15%. The recoveries were calculated by comparing the peak area ratio of ureidomustin and internal standard between pre-extraction and post-extraction spiked samples. The percentage of extraction recovery (% extraction recovery) for the lowest acceptable reproducibility concentration was defined to be within ±20%.

Stability tests of ureidomustin in various biological samples were performed at four time points after sample preparation (freeze–thaw, short-term, long-term and post-preparative, see the operational definition provided in below) at two nominal concentrations of ureidomustin (1 and 10 μg/mL). All stability studies were analyzed by spiking a standard solution with various biological matrixes. The sample preparation was similar to that described in Section 2.5.1. Freeze–thaw stability was determined after three freeze and thaw cycles. Short-term stability was assessed after being maintained at room temperature for 4 h. Long-term stability was determined after being stored at −20 °C for 30 days. Post-preparative stability was evaluated after being kept in autosampler at 4 °C for 6 h. Stability is represented as mean relative error (%) between freshly prepared samples and the samples prepared for stability, each tested by the peak area ratio of ureidomustin and internal standard. The sample stability was defined as the stability within 15% deviation of the freshly prepared samples and the samples under different conditions of freeze–thaw, short-term, long-term, and post-preparative.

2.3. LC–MS/MS operation conditions

The UPLC–MS/MS system was equipped with ACQUITY UPLC and Xevo^{TM} TQ tandem mass spectrometer with electrospray ionization (ESI, source: Waters, Manchester, UK). Chromatographic separation was achieved on a Waters BEH C18 column (2.1 mm × 50 mm, 1.7 μm) with guard-column cartridge (ACQUITY UPLC BEH C18 2.1 mm × 5 mm, 1.7 μm), and eluted with acetonitrile–0.1% (v/v) formic acid (45:55, v/v) at a flow rate of 0.3 mL/min. The column temperature was set at 40 °C. The mass spectrometer was operated in positive ion mode, combined with using multiple reaction monitoring (MRM) to monitor the mass transitions. MRM transition m/z 466.17 → 189.03 was applied to quantify ureidomustin, and m/z 181.10 → 94.99 was used for propyl paraben (internal standard). The optimized parameters of the mass spectrometer were: capillary voltage 0.5 kV, desolvation temperature 500 °C, desolvation gas 1000 L/h, source temperature 150 °C.

2.4. Experimental animals and drug administration

2.4.1. Animals

Specific pathogen-free male Sprague-Dawley rats (240 ± 30 g) were purchased from the Laboratory Animal Center of the National Yang-Ming University, Taipei, Taiwan.

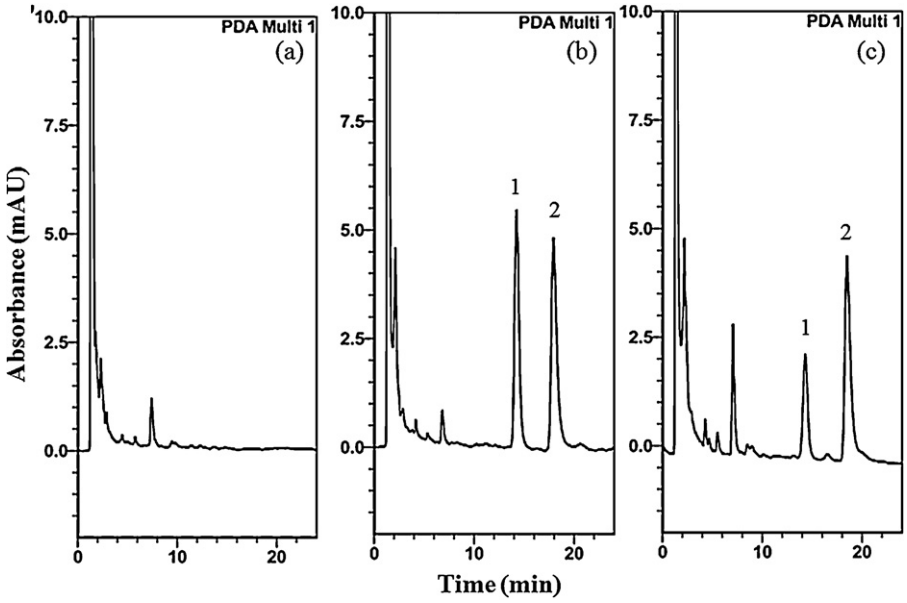

Fig. 2. HPLC-UV chromatograms of plasma ureidomustin in (a) blank plasma, (b) blank plasma spiked with ureidomustin (5 μg/mL), and (c) rat's plasma sample containing ureidomustin (2.25 μg/mL) collected at 15 min after ureidomustin administration (10 mg/kg, i.v.). 1: ureidomustin; 2: propyl paraben (internal standard).

Animals were housed in a 12/12 h light/dark cycle environment, with temperature of the colony being maintained at 24 °C and food (Laboratory rodent diet 5001, PMI Feeds, Richmond, IN, USA) and tap water being provided *ad libitum*. All procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the institutional review committee on campus.

For the drug administration and blood sampling, rats' right femoral and jugular veins were catheterized with polyethylene tube under anesthesia using a mixed solution of urethane (1 g/mL) and chloralose (0.1 g/mL) administered via intraperitoneally (i.p.) route at the dosage of 1 mL/kg. Surgical sites were pre-shaved, cleaned with 70% ethanol solution and maintained sterilely during the surgery.

2.4.2. Drug administration and plasma collection

Ureidomustin was dissolved in 0.9% normal saline and administered to anesthetized rats ($n = 5$) via intravenous (i.v.) route (the jugular vein) at a single dose (10 or 30 mg/kg). An aliquot 200 μL blood sample was obtained from right jugular vein into a heparin-rinsed tube at 5, 10, 30, 60, 90, 120, 150, 180, 240, 300, 360 and 420 min after drug administration. Each blood sample was centrifuged at 3000 × *g* for 10 min to acquire the plasma, and the plasma samples were preserved at −20 °C for further sample analysis.

2.4.3. Biodistribution

At 15 min after ureidomustin administration (10 mg/kg, i.v.), blood samples were collected by cardiac puncture and then perfused with normal saline through left ventricle. The various organs of heart, liver, spleen, lung, kidney, and brain were collected, weighed and homogenized. The biological samples were stored at −20 °C for sample analysis.

2.5. Sample preparation

2.5.1. Extraction of ureidomustin from plasma samples

To each of 70 μL of plasma sample, 14 μL of internal standard solution was added. Plasma proteins were precipitated by gentle mixing of the plasma sample with 280 μL of acetonitrile for 30 s. Then, the sample was centrifuged at 16,000 × *g* for 10 min at 4 °C. The supernatant, containing the extracted ureidomustin was transferred to an Eppendorf vial for dryness at 40 °C with a centrifugation evaporator. The dried sample was reconstituted in 70 μL of 50% acetonitrile (v/v), vortexed, and filtered by a 0.22 μm filter. The filtrate (50 μL) was transferred to autosampler vials and fixed sample loop volume (20 μL) was injected into the HPLC-PDA system.

2.5.2. Extraction of ureidomustin from organ samples

After homogenization of the organs with 50% aqueous acetonitrile solution (1:5, w/v), the homogenate was centrifuged at 16,000 × *g* for 15 min at 4 °C and the supernatant was collected and frozen at −20 °C until analysis. To determine ureidomustin level in the organs, acetonitrile (280 μL) was added to each 70 μL biological supernatant and internal standard solution (14 μL) into an Eppendorf vial for protein precipitation. The sample was then prepared similar to that in Section 2.5.1.

2.6. Pharmacokinetic applications

All pharmacokinetic analysis was evaluated using the WinNonlin Standard Edition Version 1.1 (Scientific Consulting, Apex, NC, USA). A compartmental model was utilized for data fitting and parameter estimation, and the essential pharmacokinetic model was confirmed by Akaike Information Criterion (AIC) for the best characterization. Pharmacokinetic parameter results were represented as mean ± SD. Statistical significance was determined by using a Student's *t*-test with $p < 0.05$ as the minimal level of significance.

3. Results and discussion

3.1. Quantification of ureidomustin by HPLC-PDA

Based on US FDA guideline, an internal standard was used to facilitate quantification of the target analyte(s) during method development of analysis. In order to select the suitable internal standard, we firstly survey from the PubMed and search the available chemicals in our laboratory. Seven compounds (including chloroxazone [11], quinacrine [12], procaine [13], propyl paraben

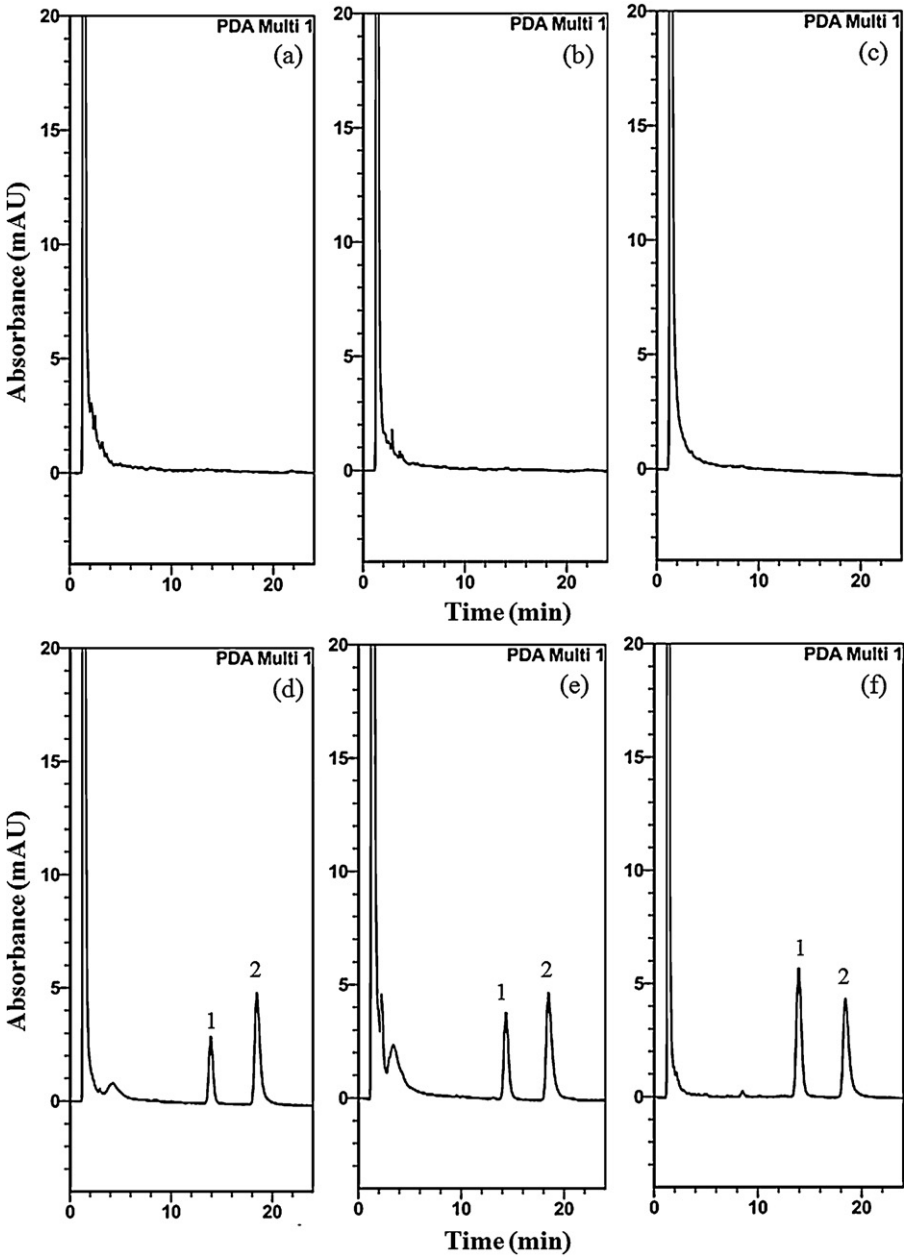

Fig. 3. HPLC chromatograms of tissue ureidomustin in (a) blank heart, (b) blank liver, (c) blank spleen, (d) rat's heart sample (8.78 μg/mL), (e) rat's liver sample (11.66 μg/mL), and (f) rat's spleen sample (18.58 μg/mL) collected at 15 min after ureidomustin administration (10 mg/kg, i.v.). 1: ureidomustin; and 2: propyl paraben (internal standard).

[14] dibutyl phthalate [15], dansyl-L-proline [16] and bis(4-nitrophenyl) phosphate (BNPP) [17]) that are structurally similar to ureidomustin have been investigated by HPLC-PDA to examine the retention time with maximum absorbance of UV wavelength at 275 nm. Among the tested compounds, propyl paraben was considered to be the most appropriate internal standard, which exhibits an appropriate retention time at 13.0 min, a maximum absorbance at the UV wavelength of 275 nm, a well resolved peak shape and was highly stable as reported by previous report [14]. Secondly, about the alternative internal standard, dibutyl phthalate may be the other candidate. Dibutyl phthalate provides the advantages of stable and common used for HPLC assay with N-mustard [15]. However, according to the above optimization to select internal standard, the retention time of dibutyl phthalate was too long and resolution was not suitable for the analysis of ureidomustin in biological sample. Based on the above optimization, propyl paraben was selected as internal standard for additional process of method validation. Typical chromatograms of the analyte and the internal standard in different biological samples are well separated and resolved as shown in Figs. 2–4. Figs. 2a, 3a–c and 4a–c show blank matrixes, and Figs. 2c, 3d–f and 4d–f show real samples after ureidomustin administration (10 mg/kg, i.v.). Propyl paraben also was selected as internal standard for analysis of nitrogen mustard-Melphalan [18].

Separation and quantification of ureidomustin blood and organs samples without other endogenous interfering peaks was achieved in an optimal mobile phase containing acetonitrile–10 mM NaH_2PO_4 (pH 3.0) (28:72, v/v) with a reversed C18 column (4.6 mm × 150 mm, particle size 5 μm). To optimize efficiency and resolution for liquid chromatography of the tested compound, we found that the pH value in mobile phase plays an important role. After replicating tests, it was found that the desirable pH value of

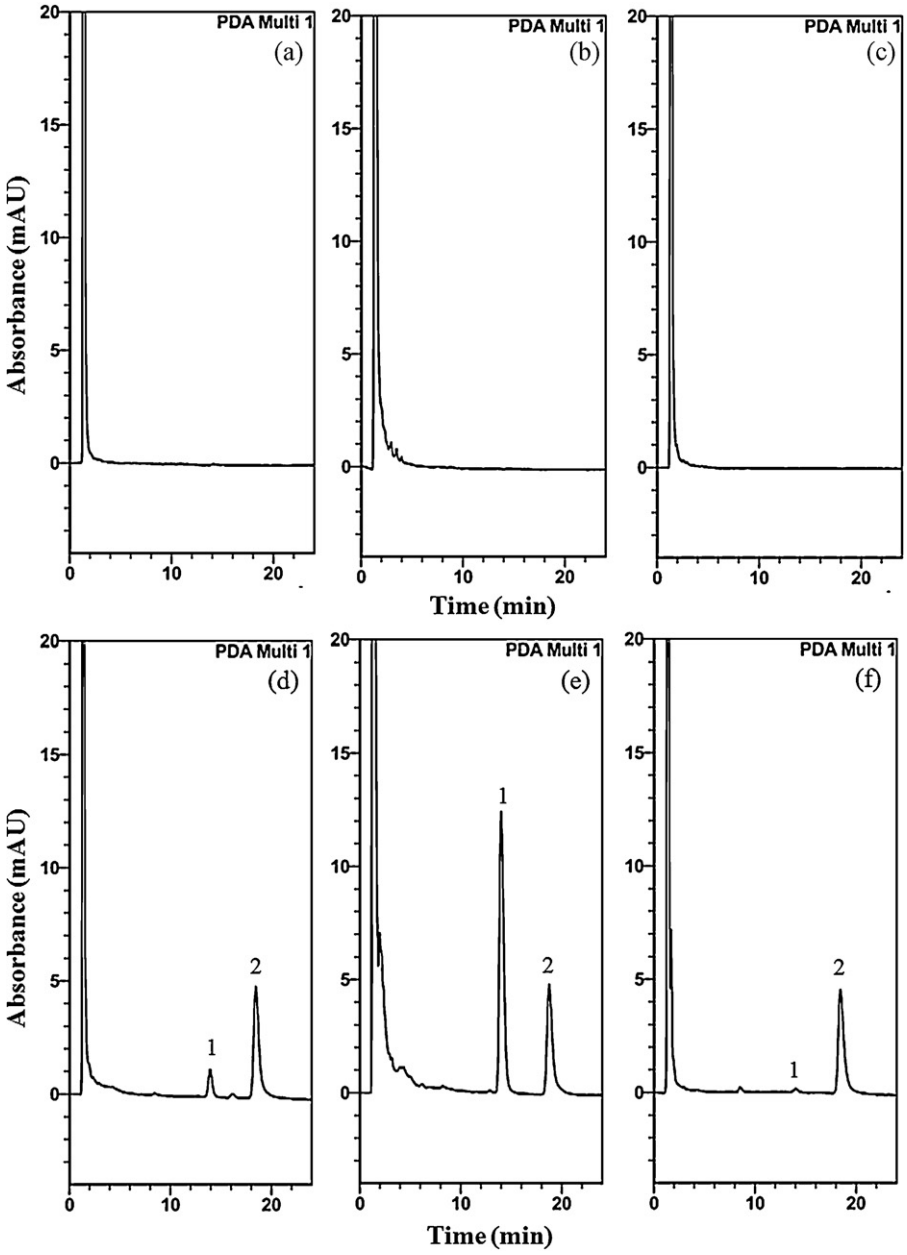

Fig. 4. HPLC chromatograms of tissue ureidomustin in (a) blank lung, (b) blank kidney, (c) blank brain, (d) rat's lung sample (3.10 μg/mL), (e) rat's kidney sample (35.86 μg/mL), and (f) rat's brain sample (2.53 μg/mL) collected at 15 min after ureidomustin administration (10 mg/kg, i.v.). 1: ureidomustin; and 2: propyl paraben (internal standard).

phosphate buffers at lower pH (pH = 3.0) could reduce the peak tailing of ureidomustin without significant endogenous interference peaks. These results show that the analytical method for ureidomustin was reproductive and available. Our results are consistent with a previous study that reported adjusting pH value in the mobile phase to be an important factor for peak shape, retention and separating efficiencies [19].

3.2. HPLC method validation

To test the linearity of ureidomustin in the biological samples, the peak area ratio of ureidomustin to internal standard vs. concentration was used. The data demonstrated that the linearity was related to concentration in the range of 0.5–50 μg/mL for plasma and 0.1–50 μg/mL for organ samples and all the coefficient correlation (r^2) were consistently greater than 0.999. The limit of detection (LOD) values for plasma and organ samples were 0.1 and 0.05 μg/mL at a signal-to-noise ratio of 3, and lower limits of quantification (LLOQ) values for plasma and organ samples were 0.5 and 0.1 μg/mL, respectively. Intra- and inter-assay accuracy (as % bias) and precision (as % RSD) value were all acceptable within one day and a consecutive day. Precision and accuracy of plasma were 1.29–13.61% and −2.40 to 3.81%, respectively, and of organ samples were 0.70–9.93% and −11.71 to 8.50%, respectively. The results are summarized in Tables 1 and 2.

To avoid the matrix effect and to enhance the extraction recovery for ureidomustin in various biological samples, solid phase extraction, liquid–liquid extraction and protein precipitation were attempted. The results showed that simple protein precipitation with acetonitrile (matrix:acetonitrile = 1:4) provides the most

Table 1
Inter- and intra-day precision (% RSD) and accuracy (% bias) using HPLC-PDA method for the determination of ureidomustin in rat's plasma.

Nominal concentration (μg/mL)	Observed concentration (μg/mL)	RSD (%)	Bias (%)
Inter-day			
0.5	0.49 ± 0.07	13.61	−2.40
1	0.99 ± 0.06	5.64	−0.88
5	5.09 ± 0.07	1.32	1.75
10	10.08 ± 0.13	1.29	0.83
50	50.37 ± 0.78	1.56	0.74
Intra-day			
0.5	0.52 ± 0.05	10.13	3.81
1	1.02 ± 0.04	4.06	2.45
5	5.05 ± 0.07	1.33	0.93
10	10.04 ± 0.17	1.72	0.43
50	50.38 ± 0.78	1.54	0.76

The data expressed as means ± SD (n = 5).

Table 2
Inter- and intra-day precision (% RSD) and accuracy (% bias) of HPLC-PDA method for the determination of ureidomustin in rat's biological samples.

Nominal concentration (μg/mL)	Observed concentration (μg/mL)	RSD (%)	Bias (%)
Heart inter-day			
0.5	0.52 ± 0.03	4.86	4.83
5	4.95 ± 0.39	7.83	−1.46
50	48.22 ± 2.78	5.77	−4.74
Intra-day			
0.5	0.49 ± 0.02	3.71	−2.87
5	4.88 ± 0.09	1.92	−3.17
50	49.72 ± 1.15	2.32	−0.74
Liver inter-day			
0.5	0.46 ± 0.03	6.26	−8.50
5	4.88 ± 0.28	5.66	6.18
50	48.97 ± 1.46	2.97	−2.06
Intra-day			
0.5	0.47 ± 0.03	6.89	−5.41
5	5.06 ± 0.20	3.93	1.18
50	48.29 ± 1.02	2.12	−4.25
Spleen inter-day			
0.5	0.44 ± 0.01	1.51	−11.05
5	4.87 ± 0.16	3.23	−1.77
50	51.91 ± 1.53	3.06	2.91
Intra-day			
0.5	0.44 ± 0.01	2.45	−11.71
5	5.32 ± 0.29	5.73	4.59
50	51.18 ± 2.13	4.26	7.89
Lung inter-day			
0.5	0.46 ± 0.03	6.94	−8.14
5	4.87 ± 0.24	5.02	−2.56
50	49.33 ± 0.69	1.41	−1.33
Intra-day			
0.5	0.46 ± 0.03	7.19	−8.63
5	4.91 ± 0.13	2.71	−1.71
50	49.03 ± 0.34	0.70	−1.95
Kidney inter-day			
0.5	0.54 ± 0.03	6.44	8.50
5	4.84 ± 0.10	2.10	−3.29
50	49.46 ± 1.21	2.46	−1.07
Intra-day			
0.5	0.53 ± 0.01	2.72	5.05
5	4.72 ± 0.20	4.16	−5.64
50	47.35 ± 2.54	5.37	−5.31
Brain inter-day			
0.5	0.48 ± 0.05	9.93	−3.00
5	4.72 ± 0.28	5.95	−7.43
50	48.45 ± 2.74	5.65	−2.76
Intra-day			
0.5	0.52 ± 0.03	6.24	4.04
5	4.90 ± 0.13	2.71	2.10
50	50.09 ± 0.42	0.84	0.17

The data expressed as means ± SD (n = 5).

Table 3
Extraction recovery (%) of ureidomustin in rat's plasma under the inter- and intra-day settings after sample preparation.

Nominal concentration (μg/mL)	Recovery (%)
Inter-day	
0.5	82.4 ± 2.0
5	82.4 ± 2.5
50	84.0 ± 4.6
Intra-day	
0.5	86.0 ± 3.2
5	83.4 ± 1.8
50	85.1 ± 2.7

The data expressed as means ± SD (n = 3).

effective extraction of ureidomustin from various biological samples. This is in accordance with a previous report which noted that modified acetonitrile protein precipitation is an effective bioassay method for HPLC-PDA [20]. Based on above test, the mean recoveries for serum and organ samples at lower (0.5 μg/mL), medium (5 μg/mL), and high (50 μg/mL) concentrations for ureidomustin were within the ranges of 83.9–91.2% (Tables 3 and 4).

Ureidomustin was quite stable under freeze–thaw cycles, short-term, as well as post-preparative stability. But this agent in different matrixes may degrade after being kept for 30 days at −20 °C, suggesting that the testing sample should be prepared as soon as possible. The results are shown in Table 5.

3.3. LC–MS/MS

To identify and reconfirm the micro amount of ureidomustin, a rapid and sensitive LC–MS/MS method was performed with an electrospray ionization (ESI) ion source capable of the positive and negative ionization mode. The results indicated that ureidomustin was sensitive when it was carried out in the positive ion mode, yielding hydrogen adduct ion $[M+H]^+$ at m/z 466.17 and transition ion of m/z 189.03. In addition, propyl paraben (ISTD) was observed with hydrogen adduct ion $[M+H]^+$ at m/z 181.10 to 94.99,

Table 4
Extraction recovery (%) of ureidomustin in rat's biological samples under the inter- and intra-day settings after sample preparation.

Nominal concentration (μg/mL)	Recovery (%)	Nominal concentration (μg/mL)	Recovery (%)
Heart inter-day		Lung inter-day	
0.5	89.3 ± 3.0	0.5	97.3 ± 6.3
5	85.9 ± 4.2	5	93.8 ± 2.9
50	87.7 ± 3.8	50	97.8 ± 6.8
Intra-day		Intra-day	
0.5	87.0 ± 2.9	0.5	92.0 ± 2.4
5	88.1 ± 2.9	5	91.8 ± 2.7
50	88.4 ± 2.3	50	93.4 ± 6.0
Liver inter-day		Kidney inter-day	
0.5	100.4 ± 0.5	0.5	89.8 ± 0.7
5	100.8 ± 7.3	5	88.5 ± 8.9
50	98.3 ± 2.9	50	86.6 ± 8.5
Intra-day		Intra-day	
0.5	98.9 ± 2.1	0.5	92.2 ± 3.8
5	100.4 ± 0.9	5	85.1 ± 3.0
50	97.0 ± 2.8	50	84.5 ± 5.0
Spleen inter-day		Brain inter-day	
0.5	81.4 ± 2.2	0.5	95.7 ± 10.2
5	80.4 ± 3.2	5	90.2 ± 4.7
50	82.2 ± 2.3	50	92.5 ± 3.4
Intra-day		Intra-day	
0.5	88.3 ± 8.0	0.5	99.0 ± 6.7
5	82.7 ± 2.2	5	97.6 ± 2.7
50	84.3 ± 3.0	50	95.0 ± 0.8

The data expressed as means ± SD (n = 3).

Table 5
Stability of ureidomustin in rat's plasma and biological samples tested at various time settings after sample preparation.

Nominal concentration (μg/mL)	Stability (%)			
	Three freeze–thaw cycle	Short-term for 4 h	Long-term for 30 days	Post-preparative stability
Heart				
1	6.26 ± 2.87	3.49 ± 1.47	−6.91 ± 3.83	−8.24 ± 0.34
10	1.69 ± 3.65	1.35 ± 0.39	−4.78 ± 1.08	−3.63 ± 1.82
Liver				
1	−8.93 ± 1.79	−4.23 ± 0.73	−4.85 ± 2.56	−8.22 ± 3.91
10	−11.13 ± 0.88	3.64 ± 1.52	−14.50 ± 1.42	−6.61 ± 1.34
Spleen				
1	10.46 ± 2.05	6.77 ± 0.65	4.55 ± 1.53	7.57 ± 0.61
10	1.57 ± 0.87	5.46 ± 2.16	−4.92 ± 1.78	2.88 ± 0.39
Lung				
1	7.92 ± 2.15	−3.85 ± 0.41	5.01 ± 2.66	9.89 ± 2.01
10	5.77 ± 2.30	1.97 ± 0.49	3.61 ± 0.35	−3.26 ± 2.88
Kidney				
1	4.76 ± 2.66	2.95 ± 0.64	9.31 ± 0.75	15.50 ± 2.69
10	5.96 ± 1.98	5.95 ± 2.06	10.33 ± 0.46	11.02 ± 0.77
Brain				
1	−3.59 ± 1.14	−12.60 ± 2.04	−9.90 ± 4.09	8.02 ± 1.69
10	−8.69 ± 1.28	−14.37 ± 2.20	−14.91 ± 4.70	−4.24 ± 3.12
Blood				
1	3.35 ± 1.51	2.69 ± 2.33	12.43 ± 1.45	10.83 ± 0.38
10	3.88 ± 0.68	10.30 ± 3.74	22.53 ± 1.15	8.20 ± 1.18

Data expressed as mean relative error ± SD ($n = 3$).

respectively. For the quantification of ureidomustin in rat's plasma, the sample extraction for LC–MS/MS analysis was carried out similarly to the process described in Section 2.5.1. Briefly speaking, plasma samples were centrifuged and then diluted to twice the volume with blank plasma, and the calibration curve was prepared by spiking the work solution with blank plasma to construct concentrations from 2.5 to 50 ng/mL. Then, sample extraction was done through acetonitrile precipitation, dryness, and reconstitution in 50% acetonitrile. The LC–MS/MS chromatograms in plasma revealed the ureidomustin peak and the internal standard at retention times of 1.08 min and 1.94 min, respectively. Fig. 5a–c shows the LC–MS/MS chromatograms in blank plasma, ureidomustin-spiked blank plasma and the rat's plasma after ureidomustin administered (10 mg/kg, i.v.). Although there existed a small interference peak at 1.15 min in blank plasma, it is of ignorant influence on the determination of peak ureidomustin in both the calibration curve range and in real biological samples. These results demonstrate that the LC–MS/MS method provides sensitive and reliable analysis. Furthermore, this analysis can be applied to validate the ureidomustin quantilization in biological samples.

3.4. Pharmacokinetics in the rats

To describe the preclinical pharmacokinetic of ureidomustin, a single bolus ureidomustin was administered via the femoral vein at two dosages (10 or 30 mg/kg) followed by a series of blood sampling

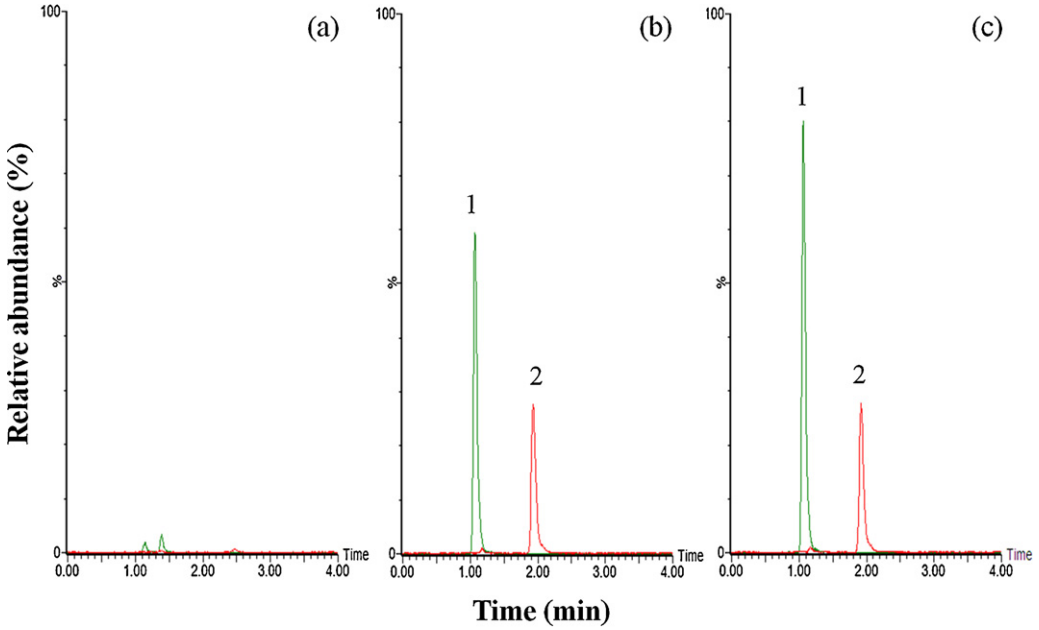

Fig. 5. LC–MS/MS chromatograms of (a) blank plasma, (b) blank plasma spiked by ureidomustin (25 ng/mL), and (c) rat's plasma sample containing ureidomustin (35.3 ng/mL) collected at 180 min after ureidomustin administration (10 mg/kg, i.v.). 1: ureidomustin; and 2: propyl paraben (internal standard).

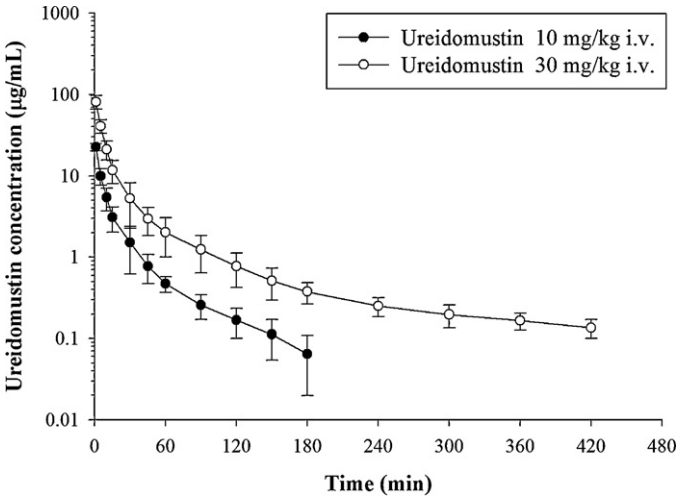

Fig. 6. Concentration vs. time profile of ureidomustin in rat's plasma after intravenous administration at a dose of 10 and 30 mg/kg. Data are expressed as means ± SD (n = 5).

at the designed time points (5, 10, 30, 60, 90, 120, 150, 180, 240, 300, 360 and 420 min after drug administration). The pharmacokinetic curves presenting the changes of ureidomustin concentrations in the plasma across time are shown in Fig. 6. The results suggested that the plasma ureidomustin was rapidly distributed and slowly decayed after ureidomustin administration in rats.

To characterize the compartmental model of ureidomustin, the AIC value was used to examine the most suitable compartment. The equation ($AIC = N \ln Re + 2p$) for minimum AIC value has been regarded as the best curve fitting for the blood concentration–time course data where N represented experimental data point, Re represented residual sum of squares for concentration difference (observed and estimated concentrations), and p represented the number of parameters in an estimated model [21]. This AIC value, in average, decreased from 25.0 ± 6.2 for the one-compartment model to 6.5 ± 16.3 for the two-compartment model at the dosage of 10 mg/kg (i.v.), indicating that the two-compartment model might be more suitable than the one-compartment model for ureidomustin administration at (10 mg/kg, i.v.). This same phenomenon is also seen with the higher dose of 30 mg/kg. However, all of pharmacokinetic parameters presented more variability in the three-compartment model. The two-compartment model is thus a more suitable pharmacokinetic model for the characterization of ureidomustin (10 and 30 mg/kg, i.v.) in the rats.

The two-compartment model is, $C_p = Ae^{-\alpha t} + Be^{-\beta t}$, where alpha ($\alpha$) and beta ($\beta$) are disposition rate constants for the distribution phase and the elimination phase, respectively. Concentrations of A and B were the concentration intercepts for the distribution phase and elimination phase. For additional interpretation of pharmacokinetic parameters, the half-life ($t_{1/2}$) was the amount of time it took for half of the drug to decay. C_0 was the initial concentration extrapolated to time zero for the i.v. dose. The area under the drug concentration–time curve (AUC) was used as a measure of the total amount of drug reaching the systemic circulation. Clearance (CL) referred to the rate at which the drug was removed from the body. Vss indicates the steady-state apparent volume of distribution. The mean residence time (MRT) was the average total time that molecules of a given dose spent in the body. The following equations represent the two-compartment model of ureidomustin at the dose of 10 mg/kg and 30 mg/kg: $C_p = 24.86e^{-0.35t} + 7.05e^{-0.05t}$ and $C_p = 82.61e^{-0.24t} + 16.38e^{-0.03t}$, respectively. The data demonstrate that the pharmacokinetic parameters for the AUC/dose (22.4 ± 5.01 vs. 28.1 ± 6.76), CL (46.9 ± 12.5 vs. 37.3 ± 8.68 mL/min/kg), Vss (711 ± 124 vs. 843 ± 362 mL/kg), and MRT (16.1 ± 5.13 vs. 23.1 ± 8.91 min) were not found to be significantly related between two groups of these groups. All the pharmacokinetic parameters of two-compartment model for ureidomustin are shown in Table 6.

Table 6
Pharmacokinetic parameters for ureidomustin administration (10 and 30 mg/kg, i.v.).

Pharmacokinetic parameters	Ureidomustin 10 mg/kg	Ureidomustin 30 mg/kg
AIC of one-compartment	25.0 ± 6.2	23.8 ± 9.7
AIC of two-compartment	6.5 ± 16.3	6.4 ± 8.1
A (μg/mL)	24.9 ± 7.77	82.6 ± 17.6**
B (μg/mL)	7.05 ± 5.00	16.4 ± 15.1
α (1/min)	0.35 ± 0.14	0.24 ± 0.09
β (1/min)	0.05 ± 0.03	0.03 ± 0.02
k (1/min)	0.14 ± 0.03	0.12 ± 0.02
$t_{1/2,\alpha}$ (min)	2.18 ± 0.76	3.24 ± 1.07
$t_{1/2,\beta}$ (min)	17.1 ± 7.11	34.1 ± 24.4
C_0 (μg/mL)	31.9 ± 4.95	99.0 ± 19.7**
AUC (min μg/mL)	224 ± 50.1	842 ± 203**
AUC/dose	22.4 ± 5.01	28.1 ± 6.76
CL (mL/min/kg)	46.9 ± 12.5	37.3 ± 8.68
Vss (mL/kg)	711 ± 124	843 ± 362
MRT (min)	16.1 ± 5.13	23.1 ± 8.91

Data are expressed as means ± SD (n = 5).
** $P < 0.01$ compared to ureidomustin (10 mg/kg, i.v.).

3.5. Biodistribution in rats

To examine the system pharmacokinetics of ureidomustin, it is necessary to relate sites of the drug's distribution in organs. The results show that this agent was quickly distributed and slowly eliminated in the rats after dosing. The analytical method for determining ureidomustin in different organ samples was same as that for plasma. In the rats (n = 5), the biodistribution of ureidomustin in the heart, liver, spleen, lung, kidney, and brain were 8.59 ± 0.80, 11.94 ± 1.31, 15.06 ± 4.19, 3.08 ± 1.64, 40.34 ± 5.07, 0.24 ± 0.02 μg/g, respectively, and in plasma was 2.11 ± 0.22 μg/mL after ureidomustin administration (10 mg/kg, i.v.). These results showed that in all tissues except the brain, there were significantly higher concentrations of ureidomustin in heart, liver, spleen, lung, and kidney at 15 min after drug administration. The highest level of this agent was found in the kidney (19-times the plasma ureidomustin level), suggesting that this water-soluble compound is eliminated mainly by kidney. The lower level of ureidomustin in the brain (11% of the plasma ureidomustin level) might be due to difficulty in crossing the blood–brain barrier.

4. Conclusion

A validated analytical method, including a simple acetonitrile sample preparation step and an accurate detection by HPLC-PDA was developed to quantitatively analyze ureidomustin in various biological samples. This method has the advantages of simplicity and high sensitivity for determining drugs in biological samples. Moreover, the current investigation also demonstrated that ureidomustin was best fitted by the two-compartment model in the SD rats. In other words, shortly after administration of ureidomustin, it was quickly distributed to various organs, accumulated primarily in the kidney and with a low concentration detected in the brain. This suggests that ureidomustin might be an effective drug for the treatment of kidney cancer, but less suitable for brain cancers. Furthermore, the developed method can help to plan an optimized drug administration cycles in chemotherapy.

Acknowledgments

Funding for this study was provided in part by research grants from the National Science Council (NSC99-2113-M-010-001-MY3, NSC99-2628-B-010-008-MY3) Taiwan, Academia Sinica (Grant No. AS-100-TP-B13) Taiwan and TCH 10001-62-007 from Taipei City Hospital, Taiwan. We thank Dr. Baker and Dr. Bessy Hung for editing on this manuscript.

References

[1] A. Gilman, F.S. Philips, Science 103 (1946) 409.
[2] P.J. McHugh, V.J. Spanswick, J.A. Hartley, Lancet Oncol. 2 (2001) 483.
[3] G.B. Bauer, L.F. Povirk, Nucleic Acids Res. 25 (1997) 1211.
[4] J.M. Kaldor, N.E. Day, K. Hemminki, Eur. J. Cancer Clin. Oncol. 24 (1988) 703.
[5] P.G. Baraldi, G. Balboni, M.G. Pavani, G. Spalluto, M.A. Tabrizi, E.D. Clercq, J. Balzarini, T. Bando, H. Sugiyama, R. Romagnoli, J. Med. Chem. 44 (2001) 2536.
[6] A.V. Patterson, D.M. Ferry, S.J. Edmunds, Y. Gu, R.S. Singleton, K. Patel, S.M. Pullen, K.O. Hicks, S.P. Syddall, G.J. Atwell, S. Yang, W.A. Denny, W.R. Wilson, Clin. Cancer Res. 13 (2007) 3922.
[7] B. Marvania, P.C. Lee, R. Chaniyara, H. Dong, S. Suman, R. Kakadiya, T.C. Chou, T.C. Lee, A. Shah, T.L. Su, Bioorg. Med. Chem. 19 (2011) 1987.
[8] C.H. Chen, Y.W. Lin, X. Zhang, T.C. Chou, T.J. Tsai, N. Kapuriya, R. Kakadiya, T.L. Su, Eur. J. Med. Chem. 44 (2009) 3056.
[9] N. Kapuriya, R. Kakadiya, H. Dong, A. Kumar, P.C. Lee, X. Zhang, T.C. Chou, T.C. Lee, C.H. Chen, K. Lam, B. Marvania, A. Shah, T.L. Su, Bioorg. Med. Chem. 19 (2011) 471.
[10] US Food and Drug Administration: Center for Drug Evaluation and Research, Guidance for Industry: Bioanalytical Method Validation, May 2001.
[11] R.F. Frye, D.D. Stiff, J. Chromatogr. B: Biomed. Appl. 686 (1996) 291.
[12] H. Ito, M. Inoue, M. Morikawa, M. Tsuboi, K. Oka, Antimicrob. Agents Chemother. 28 (1985) 192.
[13] W.W. Qin, Z. Jiao, M.K. Zhong, X.J. Shi, J. Zhang, Z.D. Li, X.Y. Cui, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 878 (2010) 1185.
[14] M.Y. Lai, S.L. Hsiu, S.Y. Tsai, Y.C. Hou, P.D. Chao, J. Pharm. Pharmacol. 55 (2003) 205.
[15] K.E. Choi, M.J. Ratain, S.F. Williams, J.A. Golick, J.C. Beschorner, L.J. Fullem, J.D. Bitran, Cancer Res. 49 (1989) 1318.
[16] M. Edler, N. Jakubowski, M. Linscheid, J. Mass Spectrom. 41 (2006) 507.
[17] J.C. Reepmeyer, J. Chromatogr. A 1085 (2005) 262.
[18] J. Rothbarth, R.W. Sparidans, J.H. Beijnen, L.J. Schultze-Kool, H. Putter, C.J. van de Velde, G.J. Mulder, J. Pharmacol. Exp. Ther. 303 (2002) 736.
[19] G. Schill, I.W. Wainer, S.A. Barkan, J. Chromatogr. 365 (1986) 73.
[20] J.C. Mathies, M.A. Austin, Clin. Chem. 26 (1980) 1760.
[21] K. Yamaoka, T. Nakagawa, T. Uno, J. Pharmacokinet. Biopharm. 6 (1978) 165.