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Stability of glufosfamide in phosphate buffers and in biological samples

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Abstract

Glufosfamide is a new, potential chemotherapeutic agent currently under investigation. Stability of glufosfamide was investigated in sodium phosphate buffers with different pH and temperature and in biological samples. Glufosfamide and isophosphamide mustard were quantified simultaneously using a liquid chromatography–ion trap mass spectrometric method; precision and accuracy were within 15% for each analyte. Glufosfamide was stable in neutral buffers, but decomposed to form isophosphoramide mustard under acidic and basic conditions, which was pH- and temperature-dependent. The stability of glufosfamide varied in different biological samples. Results indicated that glufosfamide was unstable in some biological samples, such as the small intestine, smooth muscles, pancreas and urine, especially in the small intestine homogenate, with a half-life of 1.1 h. But the pH (<8) and β-glucosidase of the tissue homogenate was found to have negligible contribution to the degradation of glufosfamide. The enzymatic inhibition experiment with the specific inhibitor, saccharo-1,4-lactone, demonstrated that it was glucuronidase that resulted in the degradation of glufosfamide in small intestine homogenate. Methanol was recommended to be used to homogenize the tissue in an ice water bath, and the container for urine collection should also be maintained in an ice water bath, and all the biological samples collected should be preserved in frozen condition until analysis.

Keywords: Stability; Glufosfamide; β-Glucosidase; β-Glucuronidase

1. Introduction

Glufosfamide, β-D-glucose-isophosphoramide mustard (Fig. 1), is a new alkylating agent. It contains an active alkylating moiety (isophosphoramide mustard) linked to β-D-glucose, that gives it the potential to exploit the trans-membrane transport system of glucose [1]. Preclinical findings suggest that cellular uptake of glufosfamide is mediated by a Na^+-dependent β-D-glucose trans-membrane transporter (SAAT1) that is followed by intracellular enzymatic cleavage of the *O*-glycosidic bond and release of isophosphoramide mustard [2]. This targeting mechanism, together with the accelerated metabolic rate and increased glucose consumption of tumor cells, suggests potentially enhanced tumor selectivity for glufosfamide and introduces a novel concept for drug targeting. In phase I and phase II clinical trials, glufosfamide was generally well-tolerated and showed anti-tumor activity against breast, colon, non-small cell lung and pancreatic cancers [3–5].

The information about the stability of drugs in biological fluids is critical for proper interpretation of analytical results. For this reason, stability assessment is considered a fundamental parameter for the validation of bioanalytical methods [6]. Since the analysis of biological samples for drug testing is usually not performed immediately after sample collection, it is very important to use suitable storage conditions in which the drug has been demonstrated to be stable during the storage time.

It was reported that glufosfamide hydrolyzes spontaneously at the glycosidic link to glucose and isophosphoramide mustard. The rate of hydrolysis depends on pH and is the fastest below pH 4. The release of isophosphoramide mustard can be accelerated by β-glucosidase [7]. However, there were no reports about the stability of glufosfamide in biological samples, which would be necessary for investigating the targeting mechanism of this anti-tumor drug. In the present experiment, to avoid the degradation of glufosfamide during sample collection, preparation and storage, the stability of glufosfamide in sodium phosphate buffers at different pH and temperature, and in different biolog-

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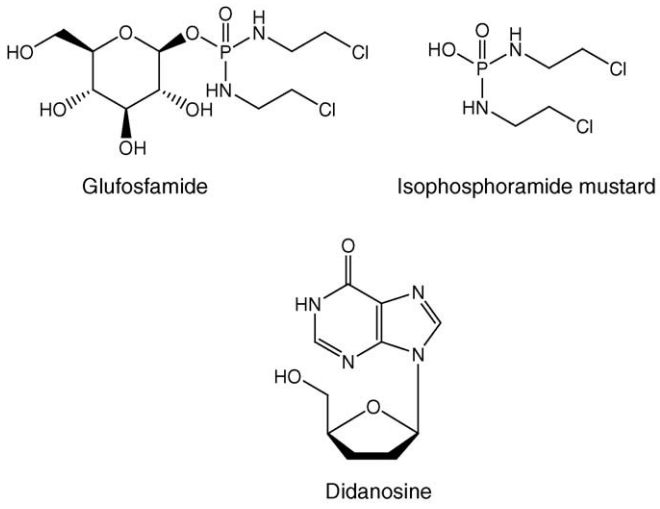

Fig. 1. Chemical structures of glufosfamide, isophosphoramide mustard and didanosine (internal standard).

ical samples at 37 °C were investigated in detail, using a liquid chromatography-ion trap mass spectrometric (LC/IT–MS–MS) method.

2. Experiment

2.1. Materials

Glufosfamide and isophosphoramide mustard were obtained from Haosen Pharmaceutical Co. Ltd. (Jiangsu, China). Didanosine for use as the internal standard (IS) was supplied by Northeast General Pharmaceutical Factory (Shenyang, China). *p*-Nitrophenyl β-D-glucopyranoside and *p*-nitrophenol were purchased from Acros Organics (New Jersey, USA), β-glucosidase, β-glucuronidase and saccharo-1,4-lactone were from Sigma (St. Louis, USA). Methanol and acetonitrile were of HPLC grade, and other chemicals were of analytical grade. Distilled water, prepared from demineralized water, was used throughout the study.

2.2. Analytical methods

An LC/IT–MS–MS method was developed for the determination of glufosfamide and isophosphoramide mustard in the stability experiment. The HPLC system consisted of a Shimadzu LC-10AD pump (Kyoto, Japan) connected to an L-column® C_{18} column (150 mm × 4.6 mm i.d., 5 μm, Waters, USA) with a C_{18} guard column (4 mm × 3.0 mm i.d., Phenomenex, Torrance, CA, USA). Isocratic chromatography was performed using the mobile phase of methanol–acetonitrile–water–formic acid (25:5:70:0.1, v/v). The flow rate was 0.5 ml/min and the column temperature was maintained at 20 °C. The injection volume was 20 μl.

A Thermo Finnigan LCQ ion trap mass spectrometer equipped with an electrospray ionization (ESI) source (San Jose, CA, USA) was used for mass analysis and detection in positive ion mode. The settings for the ESI ion source were: capillary temperature 200 °C, capillary voltage 6.0 V, and ion-spray voltage 4.5 kV. Nitrogen was used as sheath gas (1.05 l/min) and auxiliary gas (0.15 l/min). For collision-induced dissociation (CID), helium was used as collision gas (0.2 ml/min). The precursor ions were *m/z* 221 for glufosfamide $[M+H-C_6H_{10}O_5]^+$ and isophosphoramide mustard $[M+H]^+$, and *m/z* 237 for didanosine $[M+H]^+$ under the ESI mode. The collision energy was set at 30 eV for *m/z* 221 and 25 eV for *m/z* 237. Quantification was performed using selected reaction monitoring (SRM) of the transitions *m/z* 221 → 80 for glufosfamide and isophosphoramide mustard, *m/z* 237 → 137 for didanosine, respectively, with a dwell time of 0.3 s per transition.

An HPLC-UV method was also established for the determination of *p*-nitrophenol released from *p*-nitrophenyl β-D-glucopyranoside in the experiment for the measurement of β-glucosidase activity. The chromatographic analysis was performed in a Hewlett-Packard 1100 Series HPLC system (Agilent, USA) equipped with an UV detector. The analytes were chromatographed on a Zorbax XDB C_8 column (150 mm × 4.6 mm i.d., 5 μm, Agilent, USA). The mobile phase consisted of 0.05 mol/l sodium phosphate buffer–acetonitrile (61:39, v/v) at a flow rate of 1.2 ml/min. The eluate was monitored at 310 nm.

2.3. Assay validation

Calibration curves for glufosfamide and isophosphoramide mustard were prepared by analyzing calibration samples prepared by spiking the blank buffer solution or intestine homogenate (36 μl) with standard solutions (4 μl), the mixture of glufosfamide and isophosphoramide mustard, and 100 μl of the internal standard (200 μg/ml didanosine in methanol). Calibration curves for *p*-nitrophenol were prepared by analyzing calibration samples prepared by spiking the blank intestine homogenate (190 μl) with 10 μl standard solution. Quality control (QC) samples were similarly prepared by spiking QC solutions from a different weighing of the reference substance. Each analytical run included a set of calibration samples, a set of QC samples in duplicate and unknown samples.

2.4. Stability study of glufosfamide in aqueous buffers

An aliquot of 450 μl 0.1 mol/l sodium phosphate buffer at different pH of 1.5, 3.0, 5.0, 6.5, 7.4, 9.1 and 11.1 adjusted with small volumes of phosphoric acid or 11 mol/l sodium hydroxide, was preincubated at 37 °C in a water bath for 5 min, 50 μl of 2.0 mg/ml glufosfamide solution was added to a final concentration of 200 μg/ml. Each of these solutions was incubated separately at 37 °C in a water bath. According to an appropriate time schedule, typically at 0, 0.25, 0.5, 1, 2, 4 and 8 h, 40 μl of each glufosfamide solution was removed, and 100 μl of didanosine solution (200 μg/ml dissolved in methanol was immediately added and mixed. To 100 μl of the mixture, 150 μl water was added and mixed. The resultant solution was frozen at −20 °C until analysis, generally within 24 h. A duplicate experiment was performed for each pH value.

On the other hand, glufosfamide solutions (200 μg/ml) in 0.1 mol/l PBS at pH 1.5 were incubated at 0, 20, 35 and 43 °C in water bath, respectively. Then samples were prepared as described above. The experiment was performed in duplicate.

2.5. Animals and sample collection

For the in vitro stability study of glufosfamide in biological samples, male Wistar rats (200–250 g) were used, which were supplied by Lab Animal Center of Shenyang Pharmaceutical University. All rats had free access to food and water until they were sacrificed by decapitation. Fresh plasma and urine were collected below 4 °C prior to experiments. Immediately after death of the animal, the heart, lung, thymus, brain, liver, kidney, spleen, pancreas, small intestine, colon, prostate, smooth muscle, testis, marrow, bladder, stomach, esophagus and fat were removed. Following removal of fat, freshly isolated tissues were rinsed with chilled 0.9% NaCl and minced. Each tissue was added with 5 volume of chilled 0.9% NaCl (w/v) and homogenized under ice-cooling with a FJ200 homogenizer (23,000 rpm, Shanghai, China) followed by centrifugation at 1000 × *g* (10 min at 4 °C) to remove particulate material, the fatty layer floating on the top of the homogenates were discarded and the supernatants were used for the incubations.

The protein concentrations of the homogenates and other biological samples, such as fresh plasma and urine of rats, were determined by the Lowry technique [8] using standard solutions of bovine serum albumin for the calibration curve.

2.6. Measurement of β-glucosidase activity

Incubations were performed at pH 6.5 for all homogenates and other biological samples. An appropriate volume of biological sample was diluted with 0.1 mol/l sodium phosphate buffer (pH 6.5) to a volume of 90 μl to make the final protein concentration at 0.5 mg/ml, and then preincubated for 5 min at 37 °C. The reaction was started by adding 10 μl of a substrate stock solution (100 mmol/l *p*-nitrophenyl β-D-glucopyranoside in distilled water) to an initial substrate concentration of 10 mmol/l. The reaction was terminated by adding 200 μl methanol to the samples after incubation period of 0 (blank) and 15 min at 37 °C. The samples were immediately frozen at −20 °C until analysis, generally within 24 h. The samples were centrifuged at 2000 × *g* for 5 min before analyzed by the HPLC-UV method previously described. The experiment was performed in triplicate.

2.7. Stability study of glufosfamide in biological sample

Glufosfamide in biological sample at 200 μg/ml was incubated at 37 °C. With the time schedule as that for aqueous buffer, 40 μl of each glufosfamide solution was removed, and 100 μl of didanosine solution (200 μg/ml in methanol) was immediately added to each as the internal standard. The samples were frozen at −20 °C until analysis, generally within 24 h. Just before analysis, the samples were centrifuged at 2000 × *g* for 5 min; 100 μl of the supernatant was transferred to a clean tube with 150 μl water, and mixed. Glufosfamide concentrations in these samples were analyzed by the LC/IT–MS–MS method described previously. A duplicate experiment was performed for each biological sample.

2.8. Enzyme kinetics

Enzyme kinetic study was performed by incubation of glufosfamide at concentrations from 0.8 to 100 mmol/l with naive β-glucosidase. Incubations were performed as the procedure described in Section 2.6, and 0.5 unit/ml of naive β-glucosidase solution in sodium phosphate buffer (0.1 mol/l, pH 6.5) was used as the substitution of the mixture of biological sample and buffer solution. The concentration of isophosphoramide mustard released from glufosfamide was detected using the LC/IT–MS–MS method, and the experiment was performed in triplicate.

3. Results and discussion

3.1. Analytical method

The sensitivity of glufosfamide and isophosphoramide mustard to ESI was evaluated by recording the full-scan mass spectra in both positive and negative ionization modes by infusing 10 μg/ml solution via a syringe pump. Glufosfamide detection was more sensitive in negative mode than in positive mode, but isophosphoramide mustard had no significant response in negative ionization mode. To determine glufosfamide and isophosphoramide mustard simultaneously, positive ionization mode was selected. A typical full mass spectrum of glufosfamide in positive mode is shown in Fig. 2, which contains the sodium adduct ion $[M+Na]^+$ (*m/z* 405), fragment ion $[M+H-C_6H_{10}O_5]^+$ (*m/z* 221) produced in source and a small amount of protonated molecule $[M+H]^+$ (*m/z* 383). Transitions of *m/z* 405 → 243 and *m/z* 221 → 80 were used, respectively, as the quantification transition in SRM mode to investigate the linear relationship between the response and nominal concentration of glufosfamide in LC/IT–MS–MS assay. The transition of *m/z* 221 → 80 showed better linearity. However, this transition was identical to that of isophosphoramide mustard, which required the chromatographic separation of glufosfamide and isophosphoramide mustard. Methanol and acetoni-

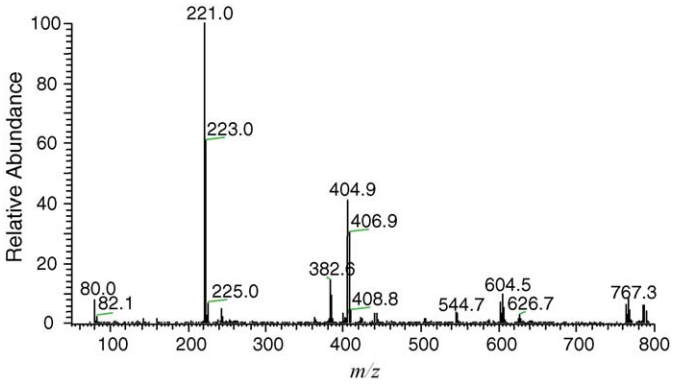

Fig. 2. Full mass spectrum of glufosfamide in positive ionization mode.

trile as organic modifier were investigated, respectively. When methanol was used alone, good peak shape but poor resolution was obtained. Acetonitrile gave good resolution but poor peak shape, when used alone as the organic modifier. Then both of them were used and the optimal constituent of the mobile phase was methanol–acetonitrile–water–formic acid 25:5:70:0.1 (v/v).

The classic analytical method reported for the measurement of β-glucosidase activity was spectrophotometry, and *p*-nitrophenol was monitored at 403 nm [10-12], where *p*-nitrophenyl β-D-glucopyranoside had no absorption. In the present study, considering the sample volume required for spectrophotometry, there was not enough homogenate for some rat tissues, such as bladder, thymus, esophagus, etc. Therefore, a validated HPLC-UV method was established and applied to the determination of *p*-nitrophenol in biological samples for the measurement of β-glucosidase activity, and the wave length for detection was set at 310 nm to improve the sensitivity.

3.2. Assay validation

Linear calibration curves for glufosfamide and isophosphoramide mustard were obtained over the range of 0.4–200 μg/ml. Typical equations of the calibration curves were as follows: glufosfamide, $y = 7.17 \times 10^{-4} + 3.94 \times 10^{-3}x$, $r = 0.9973$; isophosphoramide mustard, $y = 1.89 \times 10^{-4} + 4.72 \times 10^{-3}x$, $r = 0.9953$. The lower limit of quantification (LLOQ) for both glufosfamide and isophosphoramide mustard was 0.4 μg/ml. Accuracy and precisions were calculated based on the results of QC samples at three concentration levels of 1, 8 and 100 μg/ml ($n = 18$). In this study, the intra- and inter-day precisions in terms of relative standard deviation (R.S.D.) were less than 10.2 and 8.9% for each QC level of glufosfamide and isophosphoramide mustard, respectively. The accuracy in terms of relative error (R.E.) was within ±7.9 and ±5.6% for glufosfamide and isophosphoramide mustard, respectively. Though the LLOQ (0.4 μg/ml) for glufosfamide was not as low as methods reported in the literature [2–4,9], but sensitive enough for the determination of glufosfamide in the present study.

p-Nitrophenol was linear over the range of 0.5–100 μmol/l with correlation coefficients greater than 0.998. Accuracy and precision were calculated based on the results of QC samples at three concentration levels of 1, 10 and 80 μmol/l ($n = 6$). The LLOQ for *p*-nitrophenol was 0.5 μmol/l. The intra-day precision and accuracy were less than 7.0 and ±4.5%, respectively.

3.3. Stability in aqueous buffers

The stability of glufosfamide was investigated in sodium phosphate buffers with different pH values from 1.5 to 11.1. The corresponding degradation profile of glufosfamide is shown in Fig. 3. A pH-dependent degradation of glufosfamide was observed. In neutral conditions, glufosfamide was found to be stable, and the concentration scarcely declined within 8 h at 37 °C, but still trace amount of isophosphoramide mustard was produced. With an increase or decrease of pH from the neutral the stability of glufosfamide decreased. When the pH value of the buffer solution was lower than 3.0 or higher than 9.0,

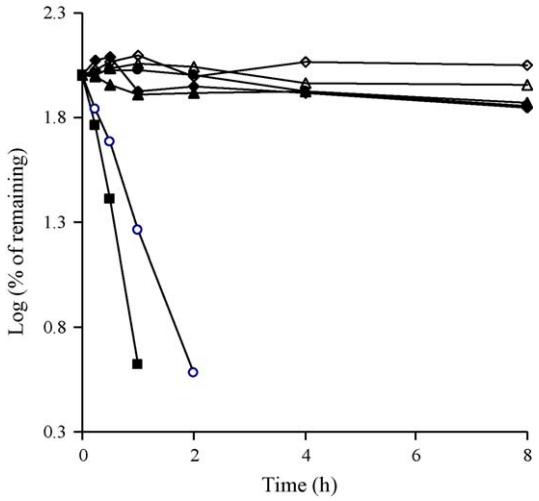

Fig. 3. Glufosfamide degradation profile in phosphate buffer solution with different pH values at 37 °C: (◯) pH 1.5, (●) pH 3.0, (△) pH 5.2, (▲) pH 6.5, (◊) pH 7.4, (♦) pH 9.0, (■) pH 11.1.

the decrease in the concentration level of glufosfamide was significant with an apparent first-order process after incubation at 37 °C. The half-lives were 25.2 and 12.6 min at pH 1.5 and 11.1, respectively. A typical chromatogram obtained from glufosfamide buffer solution at pH 1.5, containing the degradation product isophosphoramide mustard and internal standard didanosine is shown in Fig. 4A. A large amount of isophosphoramide mustard was detected simultaneously. The concentration of isophosphoramide mustard increased initially, and decreased from 1 h after incubation at pH 1.5 and 11.1 as shown in Fig. 5, due to the instability of isophosphoramide mustard as reported in the literature [13]. The degradation of glufosfamide to isophosphoramide mustard was easier in acidic and basic conditions than in neutral condition, which was similar to the hydrolysis of ester. It was proposed due to the property of ester bond possessed by the glycosidic bond existing in the structure of glufosfamide.

The effect of temperature on the stability of glufosfamide was also evaluated in buffer solutions at pH 1.5. The degradation profiles of glufosfamide at different temperatures are shown in Fig. 6. It was indicated that the decay of glufosfamide was accelerated by the increase of temperature. The Arrhenius' plot of the degradation exhibited negligible deviation from a straight line over the whole experimental temperature range ($r = 0.9992$). The results showed a temperature-dependent degradation of glufosfamide. The half-lives were 44.3, 2.15, 0.31 and 0.15 h in phosphate buffers with pH 1.5 at 0, 25, 35 and 43 °C, respectively, which indicated that temperature strongly affected the stability of glufosfamide and lower temperature can stabilize glufosfamide.

3.4. Stability in biological samples

The stability of glufosfamide in rat tissue homogenates, plasma and urine were investigated. Glufosfamide was found stable in most kinds of biological samples, except for small intestine, smooth muscle, pancreas and urine, as shown in Fig. 7. The pH value and activities of β-glucosidase were firstly proposed to

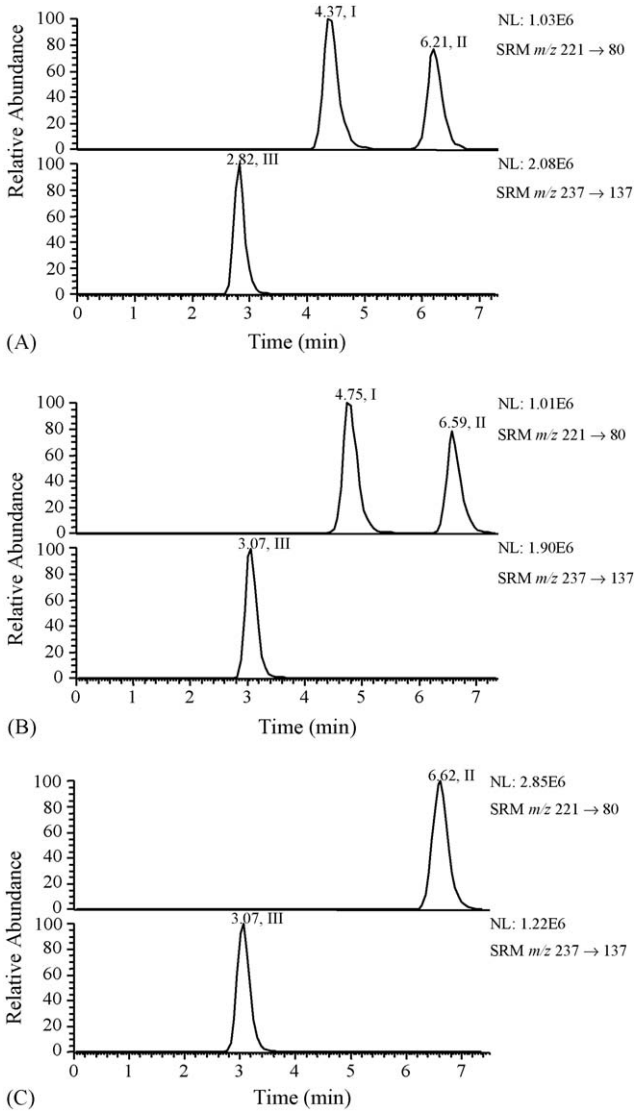

Fig. 4. Typical chromatogram of glufosfamide solution 0.5 h after incubated in buffer at pH 1.5 (A), 1 h after incubated in small intestine homogenate (B), and 3 h after incubated with small intestine homogenate added with saccharo-1,4-lactone (C), at 37 °C. I: isophosphoramide mustard, II: glufosfamide, III: didanosine.

be the possible reason for the degradation of glufosfamide in biological samples, because they were reported to have impact on the stability of glufosfamide [7]. The results described above also proved that the stability of glufosfamide was pH- and temperature-dependent. To clarify the reason, which caused the degradation of glufosfamide in partial biological samples, the pH value and β-glucosidase activity in biological samples were determined. Fresh rat urine was about at pH 9.0, the half-life of glufosfamide was 15.1 h, similar to that (12.2 h) in buffer solution at pH 9.0, which indicated that pH played an important role in the degradation of glufosfamide in urine. On the other hand, the pH values of rat homogenates and fresh plasma were near biological pH (7.4), but the half-lives of glufosfamide were 1.1, 12.7 and 14.4 h in small intestine, smooth muscle and pancreas. It was suggested that pH was not the unique factor for the degradation of glufosfamide in biological samples.

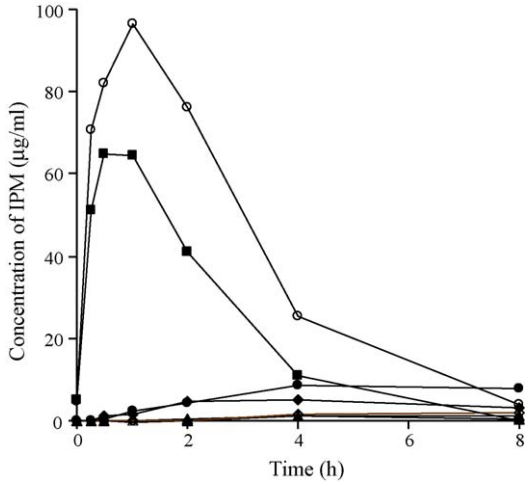

Fig. 5. The concentration profile of isophosphoramide mustard produced in glufosfamide buffer solutions: (◯) pH 1.5, (●) pH 3.0, (△) pH 5.2, (▲) pH 6.5, (◊) pH 7.4, (♦) pH 9.0, (■) pH 11.1.

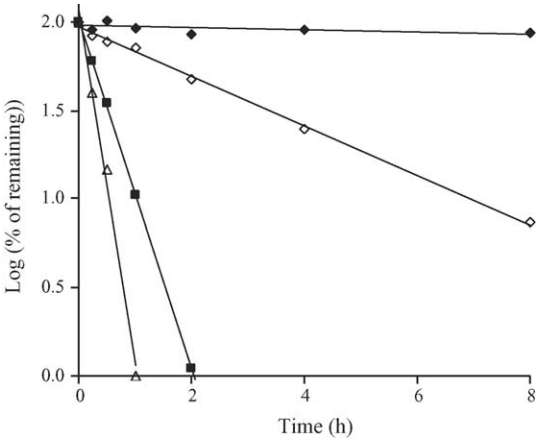

Fig. 6. Glufosfamide degradation profile in phosphate buffer solution with different temperatures at pH 1.5: (♦) 0 °C, (◊) 20 °C, (■) 35 °C, (△) 43 °C.

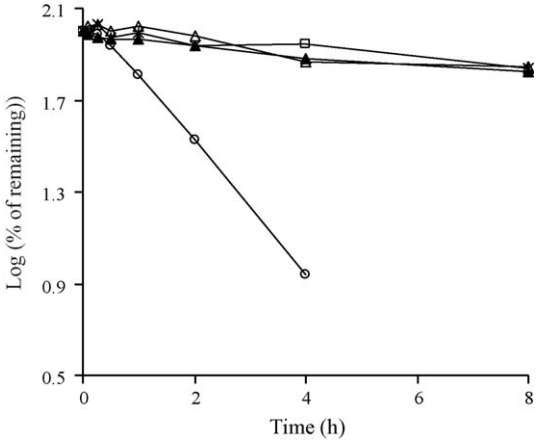

Fig. 7. Glufosfamide degradation profile in partial biological samples at 37 °C: (◯) small intestine, (△) smooth muscle, (▲) pancreas, (□) urine.

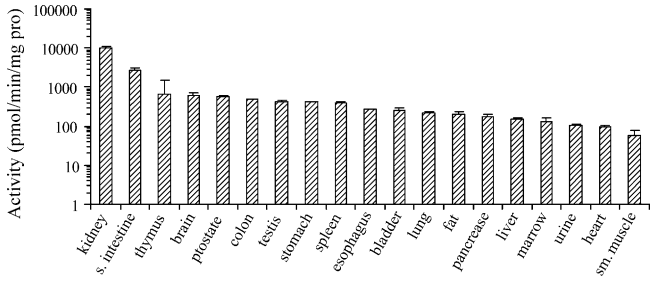

Fig. 8. β-Glucosidase activity (pmol/min/mg) for hydrolyisis of *p*-nitrophenyl β-D-glucopyranoside (10 mmol/l in 0.1 mol/l sodium phosphate at pH 6.5) in tissue homogenates (mean ± S.D., $n = 3$).

The results of β-glucosidase activity measurement in biological samples are shown in Fig. 8, and no activity was found in plasma. The relationship between the stability of glufosfamide and β-glucosidase activity in biological samples was investigated, but no correlation was found. Furthermore, glufosfamide was found to be stable in kidney homogenate, in which high β-glucosidase activity was observed. In a preliminary experiment, protein concentration was demonstrated to have no significant effect on the β-glucosidase activity in the range of 0.5–15 mg/ml. It can then be concluded that β-glucosidase had negligible contribution to the degradation of glufosfamide in biological samples. This seems to conflict with the report that β-glucosidase could accelerate the hydrolysis of glufosfamide [7]. It would be interesting to determine the susceptivity of glufosfamide to β-glucosidase. The K_m of glufosfamide was determined to be 33 mmol/l in the enzyme kinetic experiment, which reflected its low affinity to β-glucosidase. It gave reasonable interpretation for the negligible contribution of β-glucosidase to the degradation of glufosfamide in biological samples.

Fig. 4B presents a typical chromatogram of glufosfamide solution 1 h after being incubated in small intestine homogenate at 37 °C. It was found that with the decline of glufosfamide concentration, the isophosphoramide mustard level increased. There must be some other factors, which result in the cleavage of the *O*-glycosidic bond of glufosfamide in biological samples, especially in small intestine homogenate. In a previous study [14], glucuronidase was found to have the ability to hydrolyze glucoside. Therefore, the contribution of glucuronidase to the degradation of glufosfamide was focused on, and an enzymatic hydrolysis experiment was performed. Glufosfamide (200 μg/ml) was reduced by 80% 1 h after being incubated with β-glucuronidase (500 U/ml), dissolved in phosphate buffer solution of pH 6.5, at 37 °C. Saccharo-1,4-lactone, the specific inhibitor of glucuronidase, inhibited the hydrolytic reaction completely at the concentration of 250 mmol/l. Then glufosfamide was incubated with intestine homogenate which had the same concentration of saccharo-1,4-lactone added to it, and no significant amount of isophosphoramide mustard was detected within 3 h, as shown in Fig. 4C. Therefore, glucuronidase played an important role in the degradation of glufosfamide in biological samples, especially in small intestine homogenate.

It is well known that the toxic side-effects of a conventional anticancer chemotherapy are mostly caused by the lack of selectivity of cytostatic drugs against tumor target cells. This selectivity can be achieved, among others, by the use of gene therapy. One of the methods of such therapy is the enzymatic activation of a prodrug in genetically transformed cancer cells, e.g. genedirected enzyme-prodrug therapy [15]. Glufosfamide is a prodrug designed using such a prodrug/enzyme system. It is transported intact into cells by SAAT1, where the active moiety isophosphoramide mustard is thought to be released by either spontaneous hydrolysis or hydrolysis catalyzed by intracellular glucosidase [16]. The results that glufosfamide can be hydrolyzed by β-glucuronidase, indicated the activation of glufosfamide is probably catalyzed by β-glucosidase and β-glucuronidase together.

In another experiment, small intestine was homogenized with methanol, and the glufosfamide solution in the methanol homogenate was incubated at 0 °C for 8 h. Results suggested that glufosfamide was stable for at least 8 h under such conditions, and no detectable isophosphoramide mustard was found.

4. Conclusion

In the present study, to investigate the stability of glufosfamide in buffers and biological samples, an LC/IT–MS–MS method was developed for the simultaneous determination of glufosfamide and isophosphoramide mustard. With this method, glufosfamide was found to be unstable in buffers when the pH was higher than 9.0 or lower than 3.0, and the stability was pH- and temperature-dependent. Glufosfamide was unstable in some biological samples, such as small intestine, smooth muscle, pancreas and urine, especially in small intestine homogenate with a half-life of 1.1 h. But pH and β-glucosidase showed negligible contribution to the instability of glufosfamide in biological samples. Glucuronidase was demonstrated to be the virtual factor, which led to the degradation of glufosfamide in biological samples. On the other hand, glufosfamide was stable in methanol homogenate of small intestine for at least 8 h at 0 °C. Organic reagents and low temperatures were shown to have the ability to stabilize glufosfamide. To avoid the degradation of glufosfamide during the sample collection procedure, methanol can be used to homogenize tissues in an ice water bath. The container for the collection of urine samples should also be maintained in an ice water bath. All the biological samples collected should be frozen until analysis.

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