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High performance liquid chromatographic determination of *N*-butyryl glucosamine in rat plasma

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Abstract

Purpose: A high performance liquid chromatography (HPLC) method for determination in plasma of *N*-butyryl glucosamine (GLBU), a highly water-soluble compound with no chromophore was developed. *Method:* To 100 μL of plasma containing GLBU was added fucose as internal standard. GLBU and fucose were derivatized using 1-phenyl-3-methyl-5-pyrazolone in the presence of sodium hydroxide at 70 °C for 30 min. The solution was neutralized with hydrochloric acid and the excess derivatizing reagent was extracted with chloroform. The aqueous layer was injected into an isocratic HPLC system consisting of an autoinjector, a single pump and a UV detector set at 245 nm. Two different 25 cm reversed phase columns were used, a 4 and a 10 μm C_{18} columns. The mobile phase was a mixture of phosphate buffer (pH 7) and acetonitrile (80:20), which was run through a pump at a flow rate of 1.0 mL/min at ambient temperature. *Results:* Derivatized fucose and GLBU appeared 24 and 28 min, and at 34 and 37 min using 4 and 10 μm columns, respectively. The assay was linear over the range of 0.2–200 μg/mL with a limit of quantification of 0.2 and 1 μg/mL for the 4 and 10 μm columns, respectively. The method was applied to the determination of GLBU in rat plasma after oral administration of 233 mg/kg of GLBU. *Conclusion:* The present assay is precise, and accurate with sufficient sensitivity for pharmacokinetic studies following therapeutically relevant doses.

Keywords: *N*-Butyryl glucosamine; 1-Phenyl-3-methyl-5-pyrazolone; Rat plasma; High performance liquid chromatography; Glucosamine; Fucose; Post-column derivatization; C_{18} columns

1. Introduction

N-Butyryl glucosamine (GLBU) is an acylated derivative of glucosamine with a substituted butyryl group at the N atom of the amino group (Fig. 1) [1]. In a rabbit model of osteoarthritis and the rat model of streptococcal cell wall antigen, the compound has shown beneficial effects following oral doses of 20 and 200 mg/kg, respectively. The compound, therefore, is under development for therapeutic use. There is no reported analytical method for the evaluation of GLBU. The drug is highly hydrophilic hence its extraction from plasma with organic solvents is unfeasible. This renders the removal of many endogenous compounds with chemical structure similar to GLBU challenging. In addition, the drug has no chromophore hence has no light absorption at the UV range. For a similar compound, derivatization with a chromophore-containing reagent has resolved the latter problem [2]. Fortunately, similar to glucosamine, GLBU is administered in large doses. The assay sensitivity, therefore, is not a limiting step and the assay may be carried out without the need for extraction and subsequent condensation.

We have previously reported an assay for glucosamine that involves derivatization for the purpose of adding a strong UV chromophore to the compound [2]. The assay, however, was found unsuitable for GLBU as the method necessitates

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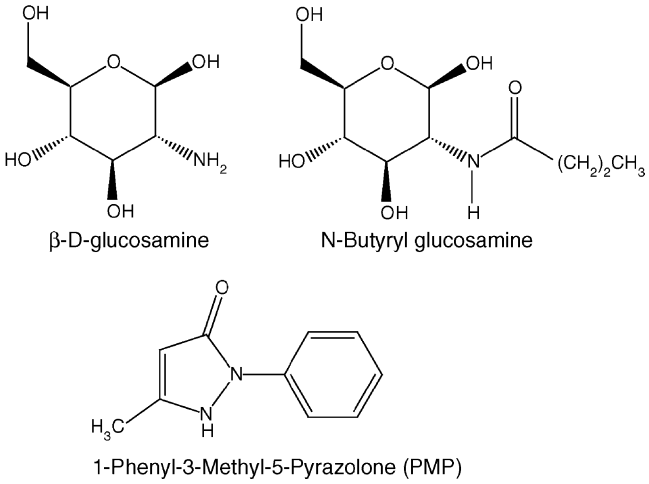

Fig. 1. Structure of β-D-glucosamine, *N*-butyryl glucosamine, 1-phenyl-3-Methyl-5-Pyrazolone.

derivatization at the N atom of an amine group. Such a group is not present in GLBU. Alternatively, hydroxyl groups, abundant in the structure of GLBU, were targeted. Such an approach is expected to result in anomeric derivatives of the carbohydrate [3–6]. Honda et al. [7] have successfully used 1-phenyl-3-methyl-5-pyrazolone (PMP) for derivatization of carbohydrates. In the present study we have described a rapid and simple HPLC method suitable for determination of GLBU in rat plasma following derivatization with PMP.

2. Experimental

2.1. Materials and reagents

D-(+)-Glucosamine butyrate was synthesized by the Department of Medicine, Queen's University (Kingston, Ont., Canada), D-(+)-fucose and 1-phenyl-3-methyl-5-pyrazolone (>95%) were purchased from Sigma Chemical Company (St. Louis, MO, USA). Methanol and acetonitrile were purchased from Caledon Laboratory Ltd (Georgetown, Ont., Canada). All chemicals and solvents were of analytical or HPLC grade. All standard solutions and mobile phase were prepared in double distilled water.

2.2. Sample preparation and derivatization

Due to hydrophilic nature of GLBU, the conventional liquid-liquid extraction of the intact drug from plasma proved unfeasible.

A stock solution of GLBU was freshly prepared by dissolving 50 mg of GLBU in 100 mL of double distilled water. Aliquots of 0.1 mL of blank rat plasma were spiked with 20 μL of fucose solution (50 μg/mL water) as internal standard and various GLBU standard solutions in 1.5 mL Eppendorf tubes to obtain concentrations of 0.2–200 μg/mL of the drug. To this solution was added 100 μL of a methanolic 0.5 M solution of PMP. The latter was premixed with an equal volume of sodium hydroxide 0.3 M. The resultant solution was heated at 70 °C for 30 min. Subsequently the solution was cooled down to room temperature and neutralized with 0.3 M hydrochloric acid. Phosphate buffer (0.2 mL, pH 2.5) was added, vortex mixed for 30 s and centrifuged at 19,000 × *g* for 1 min. The liquid part of the sample was separated from precipitated plasma residual and transferred into a 1.5 mL Eppendorf tube. To remove the excess derivatizing reagent and its degradation products as well as other unwanted compounds, the resultant aqueous solution was subjected to three times of extraction with 0.3 mL chloroform followed by vortex mixing for one min and centrifuging at 19,000 × *g* for 30 s. The upper aqueous layer, which contained GLBU was separated and 0.1 mL of it was injected into the HPLC system.

To assess the effect of addition of plasma on the recovery of GLBU, two series of triplicate samples containing 1, 10, and 20 μg/mL of the drug were prepared as described above, one with and another without plasma. To the set without plasma, water was added instead of plasma. The percent recovery was determined from

$$\%\ \text{Recovery} = \frac{\text{Peak response (plasma standard)}}{\text{Peak response (water standard)}}$$

For the assessment of precision and intra- and inter-day variation, triplicates of spiked plasma samples at concentration range of 0.2–200 μg/mL were used (Table 1).

To test the stability of derivatized GLBU and internal standard during the assay period, samples were placed a pH range of 2–8 at room temperature.

2.3. Interference with glucosamine and glucose

To determine the possibility of interference with glucose and glucosamine, aqueous solutions containing the latter compounds were subjected to the method described for GLBU. The resulting solutions were injected into the HPLC under identical conditions described for GLBU. For glucosamine, this experiment was repeated after spiking of blank plasma samples as well.

2.4. HPLC system

The HPLC system consisted of a single model M 45 HPLC pump (Waters, Mississauga, Ont., Canada), model SIL-9A autoinjector, a variable UV detector model SPD-10A UV–vis (Shimadzu, Japan) set at 245 nm and an integrator model 3390A (Hewlett Packard, USA).

Two different HPLC columns both from Phenomenex (Torrance, CA, USA) were tested, a 250 mm × 4.6 mm i.d. 10 μm of reversed-phase C_{18} and a 250 mm × 4.6 mm i.d. 4 μm of Synergi Hydreo-RP 80 Å polar endcapped C_{18} analytical columns. To both columns was attached an HPLC precolumn insert packed with C_{18} (Waters, Mississauga, Ont., Canada).

Table 1
Accuracy (% error) and precision (coefficient of variation, % CV) of the *N*-butyryl glucosamine derivative in rat plasma ($n = 3$) using a 4 μm Synergi Hydreo-RP 80 Å column

Added	Inter-day variability			Intra-day variability		
	Concentration (μg/mL)		CV (%)	Concentration (μg/mL)		CV (%)
	Observed	% Error		Observed	% Error	
0.2	0.18	9.9 ± 1.8	1.2	0.19	6.6 ± 0.6	2.9
1	0.9	9.3 ± 0.52	4.6	0.9	9.7 ± 0.86	11.4
10	9.5	4.6 ± 0.36	2.4	9.8	2.1 ± 0.21	1.3
20	19.1	4.5 ± 0.25	0.7	19.0	4.8 ± 0.32	0.8
100	98.8	1.2 ± 031	1.2	99.3	0.7 ± 0.35	4.7
200	200.8	0.6 ± 0.08	0.8	200.6	0.7 ± 009	3.3

For both columns, the mobile phase consisted of an 80:20 pH 7 phosphate buffer:acetonitrile which was pumped at a flow rate of 1.0 mL/min. The system was run at ambient temperature.

2.5. Animal study

The study protocol was approved by the University of Alberta Animal Care Committee.

One day prior to GLBU administration, silastic catheters (0.58 mm i.d. × 0.965 mm o.d.; Clay Adams, Parsippany, NJ, USA) were surgically implanted into the right jugular vein of four adult male Sprague–Dawley rats (288 ± 5 g) that were under anesthesia with halothane. The animals were allowed to recover overnight. They had free access to water and food. To administer oral doses of 233 mg/kg of GLBU, an adequate volume of an aqueous solution of 133 mg/mL GLBU was administered through a gastric gavge. Blood samples (0.3 mL) were collected from the jugular vein catheter before dosing and at 5, 10, 15, 30 min, 1, 1.5, 2, 3, and 4 h post-dose. Plasma was separated by centrifugation and stored at −20 °C until analyzed. Aliquots of 0.1 mL plasma samples were analyzed for GLBU concentrations.

2.6. Identification of the peak representing derivatized butyrylglucosamine

Sample extracts of plasma obtained from animals that had received GLBU were processed as described in Section 2.2 and inject into the HPLC system as described above. The eluent coinciding with the time of appearance of the derivatized GLBU was collected and injected into a mass spectrometer (Agilent 1100 MSD, Mississauga, Ont., Canada) with an electrospray ionization interface in positive ion mode. This experiment was also carried out on the extracts of solutions spiked with GLBU in the absence of plasma.

2.7. Peak characteristics

The peak capacity factor for both the drug and the internal standard was calculated from $K = (t_R - t_0)/t_0$ where t_R and t_0 are retention times of the peak of interest and the solvent front, respectively. The selectivity factor was estimated from $\alpha = K_2/K_1$, where K_2 and K_1 are the capacity factors of the drug and internal standard, respectively. The resolution factor was calculated from $R = 2(t_2 - t_1)/(W_2 + W_1)$, where W_1 and W_2 are base-width of the internal standard and the drug, respectively.

3. Results

Using the 10 μm C_{18} column, peaks representing the PMP derivative of the internal standard and GLBU appeared 34 and 37 min post injection, respectively. With the 4 μm Synergi Hydreo-RP column, retention times were 24 and 28 min for the internal standard and GLBU, respectively (Fig. 2). The run-time could have been shortened using a solvent–gradient system. Such a system was not available to us at the time. Many endogenous peaks were observed but none interfered with the peaks of interest. Glucosamine was also found to be derivatized with PMP but did not interfere with the drug or internal standard peaks.

Mass-spectrums obtained from the injection of the HPLC eluents in the present and absence of plasma indicated a molecular ion of 580 *m/z* for derivatized GLBU. The mass spectrum is depicted in Fig. 3. Positive ions consistent with the structure of derivatized GLBU include: 618.2 [MK^+, 30%], 602.2 [MNa^+, 95%], 580.2 [MH^+, 100%] and 510.2 [$MH^+ - CH_3\ (CH_2)_2CO$].

Using the 4 μm column, the derivatives of GLBU and internal standard exhibited capacity factors of 11.7 and 9.7, respectively. The selectivity and resolution factors between the two compounds were 1.2 and 5.1, respectively. Peaks appeared in excellent symmetrical shapes (Fig. 2).

Peaks were substantially broader with 10 μm C_{18} column as compared with the 4 μm column so that the limit of quantification of GLBU in rat plasma was 0.2 for the latter and 1 μg/mL for the former column (Table 1) using 0.1 mL of plasma sample. The detection limit, defined by a signal-to-noise ratio of 3:1, was 0.1 μg/mL for both columns. The correlation between peak area and concentration of GLBU was linear over the range of 0.2–200 μg/mL ($r^2 \geq 0.99$). For both columns calibration curves were forced through the origin

Fig. 2. Typical chromatograms obtained using a 4 μm Synergi Hydreo-RP 80 Å column of: (a) rat blank plasma, (b) blank plasma spiked with 20 μg/mL GLBU, and (c) plasma sample 10 min after oral dosing of a rat with 233 mg/kg of GLBU. Peaks: 1, 2 and 3 represent glucose, internal standard and GLBU derivatives, respectively.

with a typical concentration–response relationship described as $Y = 0.43X$, where Y was the ratio of the area under the peak of the drug over internal standard, and X was the concentration. The intra- and inter-day variation in response was <11.4% for all data points (Table 1).

Under the condition of our assay, spiked glucosamine appeared as a resolved peak that did not interfere with the peaks of GLBU or the internal standard. Since blank plasma contains endogenous glucose, we tested for the retention time of glucose in the absence of plasma. Derivatized glucose appeared 16 and 26 min using the 4 and 10 μm columns, respectively. The peak did not interfere with our peaks of interest (Fig. 2).

The presence of plasma in solutions of GLBU resulted in 30% reduction in response as the mean recovery was 70.0 ± 3.8%.

Chromatograms of blank spiked and plasma samples following administration of oral doses of 233 mg/kg to the rat are shown in Fig. 2. No glucosamine was detected following administration of GLBU.

During the method development of this assay we noticed that the size of the peak of the internal standard and, to a smaller extent, that of the drug, were decreasing with time indicating lack of stability in the final solution and/or in mobile phase during the assay. This was found to be a pH dependent effect that occurred under alkaline condition. The derivative was stable at pH 2.5 (Fig. 4).

Following oral administration, GLBU was absorbed and cleared rapidly (Fig. 5). Peak plasma concentration was attained within 45 min. Terminal phase half-life of GLBU was 1.4 ± 0.5 h. The area under the plasma concentration-time curve (0–4 h) was found to be 7.2 ± 1.2 h μg/mL.

4. Discussion

Similar to glucosamine, GLBU is a very polar compound and lacking ultraviolet absorbing properties. This makes the compound difficult to extract from plasma and detect using the common HPLC detectors. In addition, it shares many physicochemical properties with endogenous chemicals that may result in the appearance of interfering peaks. Derivatization of the drug with a suitable agent may provide a specific and UV detectable derivative. Hence, we tried to derivatize GLBU with various derivatization agents. They included naphthyl isothiocyanate, the compound that we have successfully used for pre-column derivatization of glucosamine [2]. The approach was not successful due, likely, to the fact that the amino group of GLBU was substituted with a butyryl group. In addition, the existing hydrogen atom of this new amide group is not sufficiently acidic to allow substitution with a possible nucleophilic chromophore. Alternatively, we targeted the primary and secondary hydroxyl groups of GLBU. This approach has been successfully applied to derivatize other carbohydrates [3–6]. We tried various reagents including napthyl isocyanate, fluorenylethylchloroformate and 9-fluorenone-4-carbonyl chloride in the presence of 4-dimetylaminopyridine. The derivatization was successful with these reagents, however, due to the lack of specificity, the resultant peaks interfered with some endogenous compounds in plasma. Alternatively, we used PMP. This reagent has been applied to the derivatization of aldoses, amino sugars and monosaccharides by Honda et al. [7]. These authors used the method for analysis of compounds of interest in aqueous media. The reported methods involved a time consuming step for evaporation of

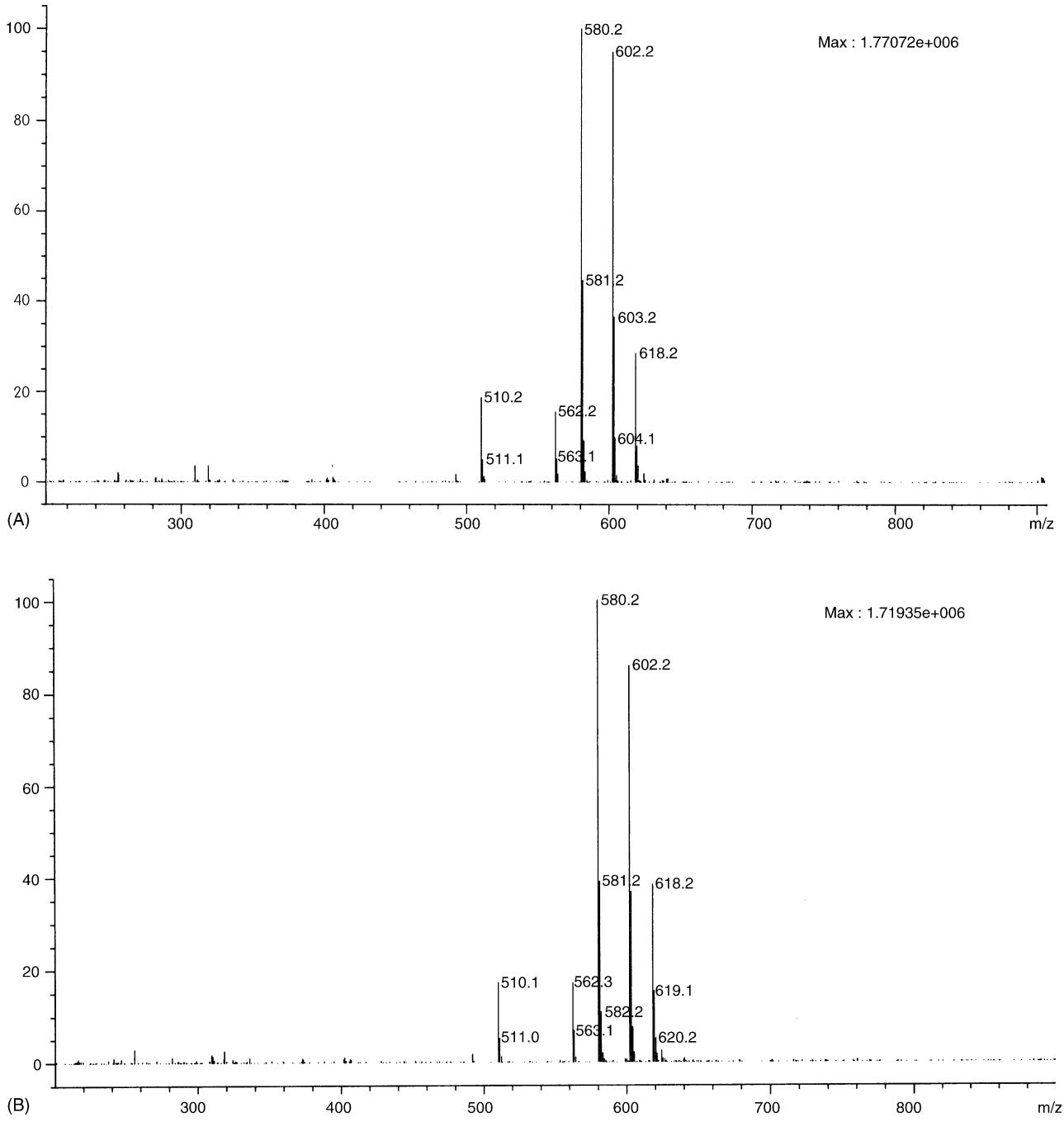

Fig. 3. Mass spectrums of eluents corresponding to the peak of derivatized butyryl glucosamine in the presence (A) and absence (B) of plasma.

aqueous solution following derivatization. We used Honda et al.'s method to derivatize GLBU in the presence of rat plasma without the need for evaporation of the aqueous solution. We found that condensation of the samples was not necessary.

Our approach involves addition of an aqueous methanolic solution of PMP to plasma samples. Tautomerism by PMP due to the presence of a keto group in the pyrazolone ring of PMP (Fig. 1) appear to result in conversion of the compound to a weak acidic enol with a low derivatization yield. Hence, there was a need to neutralize the solution with sodium hydroxide and make a sodium salt which was more reactive than the acid. The reaction may be an interaction involving the active methylene group of two molecules of the reagent and one molecule of GLBU. For glucose, it has been shown that two molecules of PMP react with one molecule of the sugar [8]. The mass spectrum presented in Fig. 3 confirms such a structure for the derivatized GLBU.

Interference by endogenous peaks was a significant problem in developing this assay. This was expected since GLBU is a carbohydrate with a chemical structure similar to

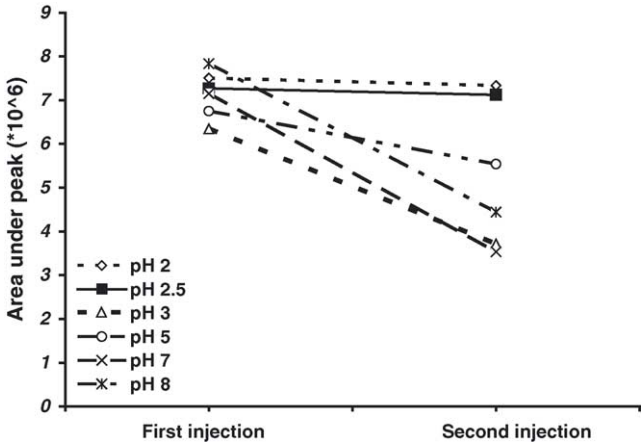

Fig. 4. Stability of internal standard derivative at room temperature over time (approximately 6 h) at different pH values.

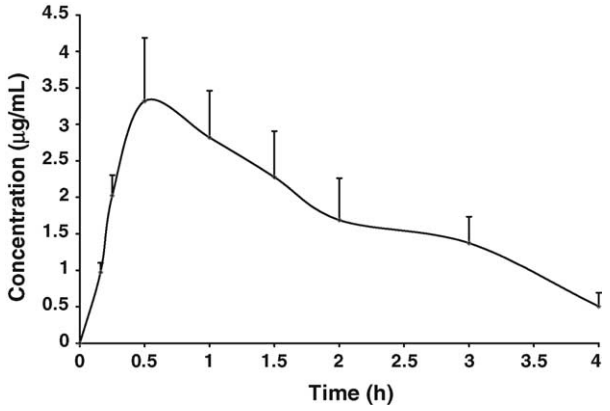

Fig. 5. Mean (±S.D.) plasma concentration vs. time profile of GLBU in rat plasma following oral administration of 233 mg/kg of GLBU ($n = 4$).

many endogenous compounds. However, optimization of the method permitted analysis of the rat plasma samples without interference. As shown in Figs. 2, the endogenous components of the rat plasma eluted before the derivatives of both GLBU and the internal standard so that they did not interfere with the peaks of interest.

PMP derivatives are not stable at high temperatures [8]. Our data indicates that this lack of stability is pH-dependent (Fig. 4). In our hands, solutions of fucose-PMP and, to a smaller extent, those of GLBU-PMP, were not stable at room temperature at pH > 2.5. Indeed, when pH 7 solutions were placed in the authosampler for injection a significant loss of peak area was noticed, particularly for samples injected later during the process. At pH equal or lower than 2.5, however, the peaks were stable. Hence, we prepared our samples at pH 2.5. The mechanism involved for these losses of response is unclear. One explanation could be the possibility of more internal hydrogen bonding formation under acidic conditions as compared with alkaline pH [7].

After oral administration of a relatively high single dose, maximum plasma concentrations ranging from 2.5 to 5.0 μg/mL were observed (Fig. 5). These relatively low peak concentrations are suggestive of either low bioavailability and/or high volume of distribution of GLBU. Further studies are needed for clarification. For glucosamine, which is also administered in large oral doses, pre-systemic loss of the drug in the gut has been suggested [9]. GLBU administration did not result in appearance of glucosamine peaks in plasma. This indicates that GLBU does not appear to bio-convert to glucosamine.

The method is specific and has sufficient sensitivity for pharmacokinetic studies of GLBU following administration of oral doses of 233 mg/kg to the rat.

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