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Microanalysis of oligosaccharide HS203 in beagle dog plasma by postcolumn fluorescence derivatization method

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ABSTRACT

A rapid and sensitive postcolumn fluorescence derivatization method was developed for microanalysis of antidiabetic oligosaccharide HS203 in beagle dog plasma. After plasma protein was removed by a simple and fast ultrafiltration method, chromatographic separation was performed on an Asahipak GS-320 HQ column with a mobile phase of 50 mmol/L phosphate buffer (pH 6.7) and acetonitrile (83/17, v/v). The column effluent was monitored by fluorescence detection at 249 nm (excitation) and 435 nm (emission) using guanidine hydrochloride as a postcolumn derivatizing reagent. A satisfactory resolution of the analyte was achieved and the limit of detection was found to be 4 ng (more sensitive than silver staining of HS203 in polyacrylamide gel electrophoresis). The method described above was successfully applied to a pharmacokinetic study of HS203 and to monitor blood glucose level simultaneously in beagle dog. It is also possible to be applied for microanalysis of other oligosaccharides in biological samples.

1. Introduction

The prevalence of type 2 diabetes mellitus (DMT2) is increasing rapidly and becoming a major world health threat in the future (Ahmad et al., 2011). The islet amyloid deposition is a characteristic pathological feature of the pancreas in DMT2 (Marzban & Verchere, 2004). Evidences show that aggregated islet amyloid polypeptide (IAPP) plays an important role in the development of beta-cell failure (Höppener & Lips, 2006). HS203 is a marine oligosaccharide complex of chromium (III) and oligomannuronic acid which was isolated from brown algae *Laminaria japonica*. The weight average molecular weight of HS203 is 3000 ± 500 Da and the distribution width of molecular weight is about 1.2. Systematic pharmacological results showed that HS203 not only had significant inhibitory activity on the fibril formation of IAPP and stimulatory activity on insulin secretion of RIN-5F cell in vitro, but also significantly improved insulin resistance in DMT2 rats and mouse (Hao et al., 2011; Zhang et al., 2008; Zhao, Yu, Guan, Li, & Hao, 2010). For further research of HS203 as a promising candidate for the treatment of DMT2, the microanalysis of HS203 in biological samples is very important to explore its physiological role, pharmacokinetics and bioavailability. However, the quantitative microdetermination of HS203 is a great challenge due to its few chromophoric and fluorophoric groups. A fluorescence prelabeling method commonly used in analysis of carbohydrates (Anumula, 2006; Harvey, 2011; Lamari, Kuhn, & Karamanos, 2003) has high sensitivity, but the polysaccharides may undergo isomerization, degradation, or conformation change and the labeling procedure is often complicated and time-consuming (Han, Lv, Jiang, & Wang, 2007). In contrast, postcolumn derivatization is naturally automatic and convenient, and plays an important role in the automation of carbohydrate analysis (Caceres, Cardenas, Gallego, Rodriguez, & Valcarcel, 2000).

In this study, we firstly present a new high performance liquid chromatographic postcolumn fluorescence derivatization (HPLC-PC) method using guanidine as a fluorometric reagent for the determination of HS203 in beagle dog blood plasma. The fast and sensitive method with a simple pretreatment procedure was successfully applied to a pharmacokinetic study of HS203 in beagle dog, and a polyacrylamide gel electrophoresis (PAGE) method was used to demonstrate this newly established procedure.

2. Experimental

2.1. Materials

HS203 (minimum purity, 99%) was provided by School of Medicine and Pharmacy (Qingdao, Ocean University of China). The beagle dog plasma samples were provided by Shandong University

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(Jinan, China). Guanidine hydrochloride and D-glucuronic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetonitrile (HPLC grade) was purchased from Merck KGaA (Germany). Ultra-0.5 centrifugal filters (30 kDa, MWCO) were purchased from Millipore Corporation (USA). All other chemicals were of analytical reagent grade.

2.2. Instrumental

The HPLC-PC system was equipped with an Agilent 1100 G1311A binary pump, a G1313A autosampler, a G1321A fluorescence detector and a system controller (all from Agilent, USA), and a PCX5200 postcolumn derivatization instrument (Pickering Laboratories). Chromatographic separation was carried out on an Asahipak GS-320 HQ (300 mm × 7.6 mm, with GS-2G7B guard column, 50 mm × 7.6 mm) purchased from Shodex Corporation (Tokyo, Japan). The mobile phase was 50 mmol/L phosphate buffer (pH 6.7) and acetonitrile in a ratio of 83:17 (v/v, %) at a flow rate of 0.5 mL/min. In the postcolumn procedure, a 0.5 mol/L sodium hydroxide solution containing 200 mmol/L guanidine hydrochloride was added to the effluent at a flow rate of 0.3 mL/min. The mixture passed through a polytetrafluoroethylene (PTFE) reaction coil (0.5 mm i.d., 10 m) thermostated at 125 °C, then a 0.5 mol/L sodium hydroxide solution at a flow rate of 0.3 mL/min was added to cool down the mixture in another PTFE coil (0.25 mm i.d., 3 m) at ambient temperature and detected at 249 nm (excitation) and 435 nm (emission).

2.3. Preparation of calibration standards and quality control samples

The stock solutions of HS203 (4 mg/mL) and internal standard (1 mg/mL) were freshly prepared in water and 1 mol/L sodium chloride solution, respectively, and were stored in a refrigerator at 4 °C before use. Working solutions of HS203 at desired concentrations were prepared by serial dilution of the stock solution with water, and the internal standard solution was diluted to 100 μg/mL with 1 mol/L sodium chloride solution. Calibration standards were prepared by spiking 50 μL of the appropriate standard solution and 50 μL of internal standard solution to 100 μL of blank dog plasma. The quality control (QC) samples were prepared at low (25 μg/mL), middle (50 μg/mL), and high (100 μg/mL) concentrations in the same way as the plasma samples for calibration, and stored at −20 °C until analysis.

2.4. Sample preparations

Plasma samples were thawed at room temperature before processing. To a 100 μL portion of plasma sample, 50 μL of internal standard solution and 50 μL of water were added, followed by vortex for 1 min, and then were transferred to a 30 kDa Ultra-0.5 centrifugal filter device. After centrifugation at 10,000 × *g* for 10 min, the aliquot (20 μL) of the subnatant was submitted to the HPLC system.

2.5. Method validation

2.5.1. Linearity

To a 100 μL portion of blank plasma, 50 μL of internal standard solution and 50 μL of working solution were added to provide the final concentrations of 1, 2.5, 5, 10, 25, 50, 100, 150 μg/mL of HS203, and then processed as described above. Calibration curves were constructed by plotting the peak area ratios of HS203 to internal standard against the concentrations of HS203. One calibration curve was constructed on each analysis day using freshly prepared calibration standards.

2.5.2. Recovery and the limit of detection

The percentage recovery was calculated by comparing the peak area ratios of extracted samples with samples in which the compound was spiked directly in mobile phase. Recoveries at three QC concentration levels for plasma were examined at least five times. The limit of detection (LOD) is defined as the lowest concentration level resulting to a signal-to-noise ratio of 3:1.

2.5.3. Precision and accuracy

For intra- and inter-day precisions and accuracies, the samples ($n = 5$) that had been spiked at concentrations of 25, 50, and 100 μg/mL for HS203 were assayed. Intra-day accuracy and precision were evaluated from replicate analysis ($n = 5$) of samples at different concentrations on the same day. Inter-day accuracy and precision were evaluated from the analysis of the same samples on five continuous days in a week ($n = 5$).

2.6. Stability studies

Three HS203 concentrations (25, 50, 100 μg/mL) were stored at 4 or −20 °C for five days to evaluate its long-term stability in plasma. Thereafter, samples were analyzed as described above. The concentrations thus obtained were compared with the theoretical value of samples to determine the long-term stability of HS203 in dog plasma.

2.7. Pharmacokinetics of HS203

Beagle dogs were used for the pharmacokinetic study of intravenously administered HS203. After administration of a single dose (60 mg/kg), blood samples were withdrawn from the femoral veins of the animals using a puncture needle at 0, 5, 15, 30, 45, 60, 120, 180, 240, 300, 360, 480 min. Plasma samples were immediately separated by centrifugation at 10,000 × *g* for 10 min, then transferred to suitably labeled tubes, and stored at −20 °C until used. Pharmacokinetic analysis of HS203 concentration in plasma was performed using the practical pharmacokinetic program (3p87) (Chinese Society of Mathematical Pharmacology). T_{max} and C_{max} values were recorded directly from the measured data.

The postcolumn derivatization procedure described above was validated by a PAGE method. The PAGE was performed on a vertical slab (0.1 cm × 8 cm × 10 cm) gel system using 22% polyacrylamide as continuous gel (Higashi et al., 2012). The gel was loaded with 10 μL of the treated plasma sample, and subjected to electrophoresis at 200 V for 2 h after pre-running at 100 V for 10 min. After electrophoresis, fix the gel in silver-ammonia solution (4 mol/L silver nitrate:0.76 mol/L sodium hydroxide:27% ammonium hydroxide:water = 5:5:7:483) for 5 min. After the gel was soaked in developer (2.5% citric acid:38% formalin:water = 1:1:500) for about 3 min, the reaction was stopped with the stop solution (acetic acid:methanol:water = 1:4:15).

3. Results and discussion

3.1. Selection of internal standard and sample pretreatment procedures

Since HS203 is a kind of uronic acid derivative, glucuronic acid was selected as internal standard due to its high recovery, suitable retention time and similar structure to our analyte. In the pretreatment procedure, HS203 was released completely from blood plasma by addition of 1 mol/L sodium chloride solution, and almost all plasma proteins were retained in Ultra-0.5 centrifugal filter (30 kDa, MWCO). The method involving centrifugal ultrafiltration was convenient, reproducible and high recoverable, and the endogenous substances in plasma did not interfere the detection of

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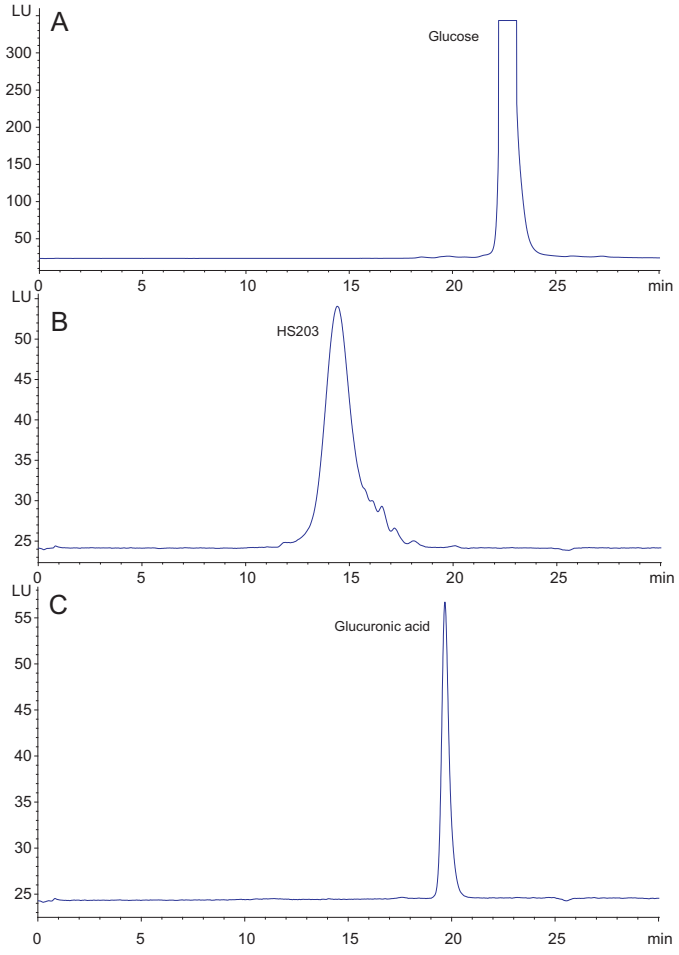

Fig. 1. Chromatograms of (A) blank dog plasma, (B) 25 μg/mL HS203, and (C) 25 μg/mL internal standard (glucuronic acid), the gain of fluorescence detector was set at 14.

HS203 in HPLC-PC analysis (Fig. 1). We have firstly used a column (300 mm × 7.6 mm) of Asahipak GS-320 HQ based on size exclusion, distribution and ion-exchange modes for the separation of HS203 in plasma. The analyte was eluted at the mobile phase conditions of a mixture of 50 mmol/L phosphate buffer (pH 6.7) and acetonitrile in a ratio of 83:17 (v/v, %) (Dai et al., 2010), which provided satisfactory resolution of HS203 and glucuronic acid. More importantly, the glucose in plasma was separated excellently with HS203 and internal standard (as shown in Fig. 1), and it provided a base for monitoring of blood sugar level after drug administration.

3.2. Optimization of derivatization conditions

To explore high sensitivity detection method for HS203, we have tested three fluorescence reagents: guanidine hydrochloride (Han et al., 2007), cyanoacetamide (Kusano et al., 2007; Toyoda, Muraki, Imanari, & Kinoshita-Toyoda, 2011) and arginine (Honda, 1996). The aldehyde group of reducing carbohydrate can react with guanidine group in alkaline medium to form a strong fluorescence cyclic compound. In the comparison study, guanidine hydrochloride offers the most sensitivity (two times more than that of cyanoacetamide) required for the samples available in limited amounts.

The concentrations of guanidine hydrochloride, reaction temperature, and concentrations of sodium hydroxide were independently varied in turn. The effects of these parameters on the peak areas of HS203 were studied and shown in Fig. 2. An array of concentrations of guanidine hydrochloride (50, 100, 150, 200 mmol/L) was individually used to label HS203 while the other factors were kept unchanged in the derivatization method, and the highest fluorescence intensity of derivatives was obtained at the concentration of 200 mmol/L (Fig. 2A). When the reaction temperature increased, the fluorescence intensity increased. The influence of temperature on the fluorescence intensity of derivatives was not significant when temperature was above 125 °C (Fig. 2B). Taking into account the influence of high temperature on the postcolumn derivatization reaction coil strength, we chose 125 °C for the optimal reaction temperature. The concentrations of sodium hydroxide had little influence on the fluorescence intensity of derivatives (Fig. 2C). To avoid the impairment of alkaline solutions on the optical properties of the flow cell of fluorescence detector, we selected 0.5 mol/L of sodium hydroxide solution as the optimal concentration.

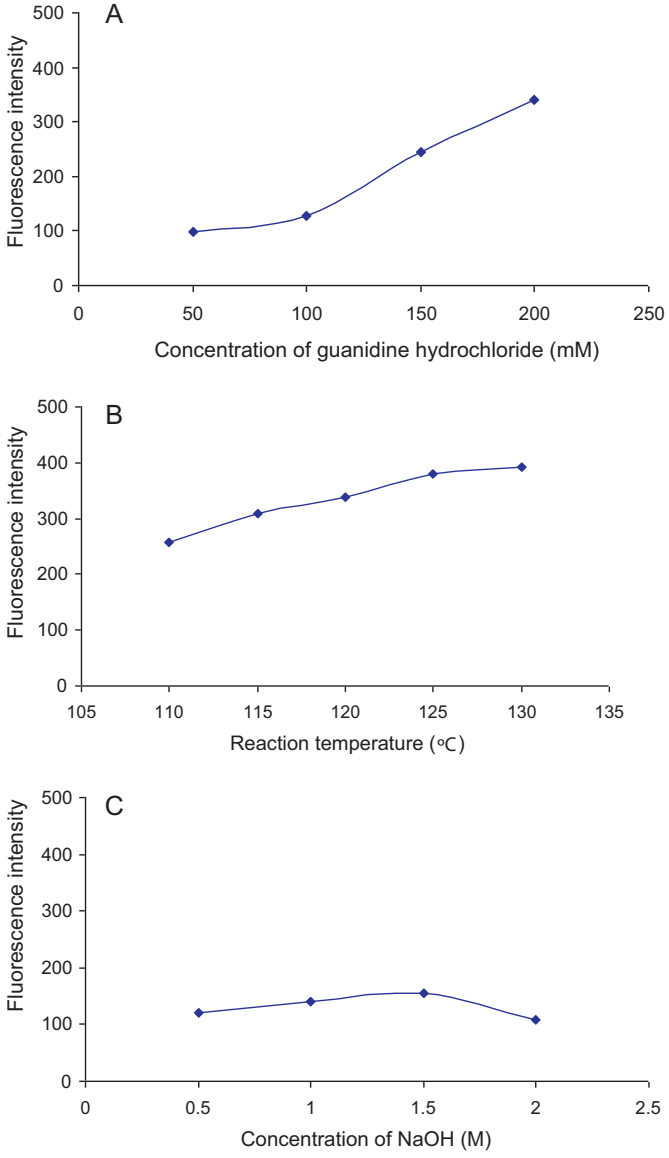

Fig. 2. Effects of (A) concentration of guanidine hydrochloride, (B) reaction temperature, and (C) concentration of sodium hydroxide on the fluorescence intensity of HS203 with guanidine hydrochloride.

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Table 1
Intra- and inter-day precision and accuracy of HS203 in dog plasma ($n = 5$).

Concentration (μg/ml)	Mean ± S.D.	Precision (%CV)	Accuracy (%bias)
Intra-day			
25	22.98 ± 0.20	0.85	8.08
50	55.57 ± 0.34	0.61	−11.14
100	113.94 ± 0.71	0.62	−13.94
Inter-day			
25	23.74 ± 0.44	1.83	5.05
50	48.02 ± 1.49	3.09	3.95
100	101.77 ± 3.55	3.49	−1.77

Table 2
Stability of HS203 in dog plasma at different temperature ($n = 5$).

T (°C)	Concentration (μg/mL)	Mean ± S.D.	%CV	%Bias
4 °C	25	25.11 ± 0.87	3.45	−0.43
	50	55.42 ± 1.92	3.46	−10.85
	100	103.39 ± 2.13	2.06	−3.39
−20 °C	25	26.69 ± 1.18	4.42	−6.77
	50	49.52 ± 0.99	2.01	0.96
	100	111.40 ± 3.67	3.29	−11.40

3.3. Method validation

3.3.1. Linearity and range

The linearity of the method was evaluated using calibration samples of eight different concentrations of HS203 (1, 2.5, 5, 10, 25, 50, 100, 150 μg/mL of HS203) in drug-free plasma. The linear correlation between the peak area ratios of HS203 to glucuronic acid and the concentrations of HS203 was excellent, from 1 to 150 μg/mL ($R = 0.9979$, $n = 8$).

3.3.2. Recovery and the limit of detection

The extraction recoveries of HS203 obtained from plasma were 92.82 ± 2.09%, 109.33 ± 4.09%, and 105.16 ± 2.62% for samples at low concentration, middle concentration and high concentration, respectively. The LOD for HS203 was 4 ng (signal-to-noise ratio = 3, gain = 17).

3.3.3. Precision and accuracy

The standard deviations of reproducibility on intra- and inter-day analyses were also satisfactory (Table 1). The intra-day CV% for three HS203 concentrations (25, 50, 100 μg/mL) was 0.85, 0.61, 0.62 ($n = 5$), respectively. In addition, the inter-day CV% for the three respective HS203 concentrations was 1.83, 3.09, 3.49 ($n = 5$), respectively.

3.3.4. Stability studies

The long-term stability data for HS203 in plasma samples stored at 4 or −20 °C were summarized in Table 2. The stability study of HS203 in dog plasma showed reliable stability behavior, as the means of the results of tested samples were within the acceptable criteria of ±15%.

3.4. Pharmacokinetics of HS203

The proposed method was applied to the quantitative determination of HS203 in plasma samples obtained from beagle dogs. Plasma concentrations of HS203 measured by HPLC-PC method were detectable up to 5 h (3.3 ± 0.5 μg/mL) after a single intravenous injection of 60 mg/kg of HS203 (Fig. 3). HS203 was readily absorbed and reached the maximum concentration of 252.9 ± 20.8 μg/mL at 5 min after intravenous administration. According to the lowest value of the Akaike's Information Criterion (AIC) implemented in 3p87, the data sets were fit to a two-compartment model. The distribution half life ($t_{1/2}\alpha$) was concluded to be 10.7 ± 0.6 min and the area under the curve (AUC) of HS203 was calculated as 10,287.3 ± 894.4 (μg min/mL). In addition, the glucose in plasma was also detected using present HPLC-PC method, and the blood sugar reached the lowest level at 2 h after intravenous administration of HS203 (Fig. 4).

The HPLC-PC method was previously reported for pharmacokinetic studies of other polysaccharides, such as chondroitin sulfate and chitosan ester, however, the obtained data about pharmacokinetic properties of original drug were not enough. Sakai et al. (2002) degraded chondroitin sulfate into unsaturated disaccharides before detection, so the metabolic activity of the original chondroitin sulfate in vivo was not traced. Han et al. (2007) determined the original chitosan ester in rabbit serum by HPLC-PC method, but the derivative only provided a single peak performing on a C8 reversed-phase column, the information of the polymerization degree variations of chitosan ester was not obtained. Conversely, we not only established the plasma concentration–time curve of the original HS203 (Fig. 3) in beagle dog, but also obtained the chromatography of oligosaccharides with different polymerization degrees in HS203 as shown in Fig. 4. From the peak height ratios of low polymerization degree oligosaccharides to the main peak of HS203 at different times after intravenous administration, we found that the low polymerization degree oligosaccharides in HS203 increased with the increasing time, for example, the peak height ratio of tetrasaccharide to the main peak increased from 0.20 to 0.32 within an hour, indicating that HS203 was degraded slightly in plasma. More importantly, the change of blood glucose level was also shown

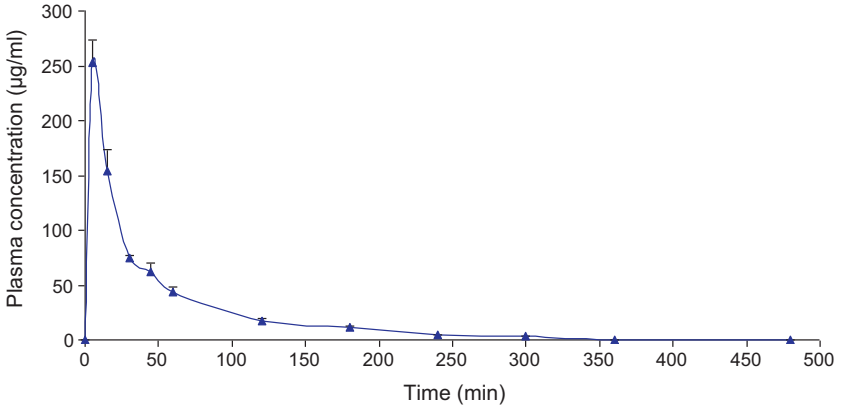

Fig. 3. Plasma concentration–time disappearance curve of HS203 in beagle dog following i.v. administration of 60 mg/kg, each data point represents the mean ± standard deviation ($n = 3$).

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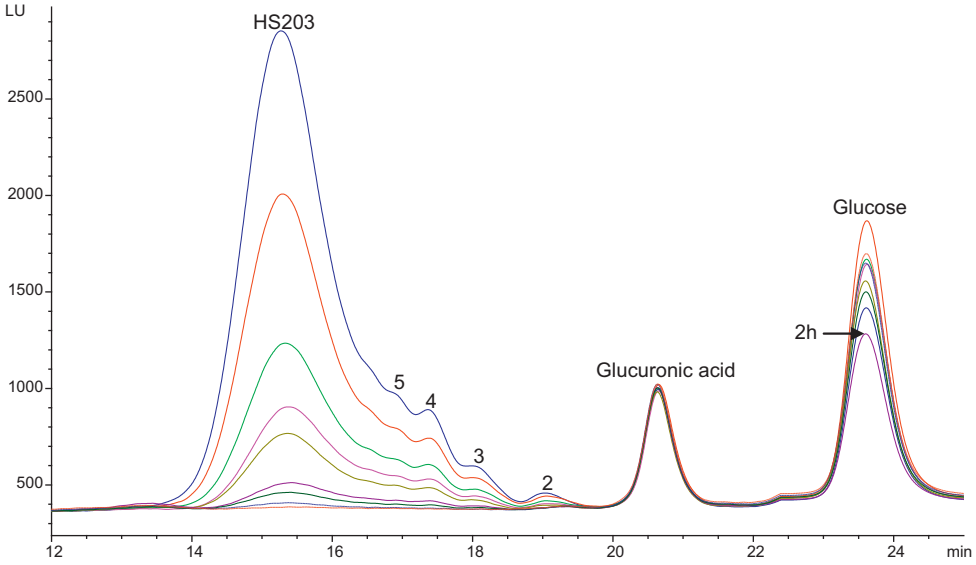

Fig. 4. The chromatograph of HS203 in beagle dog following i.v. administration of 60 mg/kg (from top to bottom: 5, 15, 30, 45, 60, 120, 180, 240, 300 min, respectively). The numbers on the peaks are the degrees of polymerization of oligosaccharides in HS203 (2-disaccharide, 3-trisaccharide, 4-tetrasaccharide, and 5-pentosaccharide). The gain of fluorescence detector was set at 17 from 0 to 22.5 min and 13 from 22.5 to 27 min, respectively.

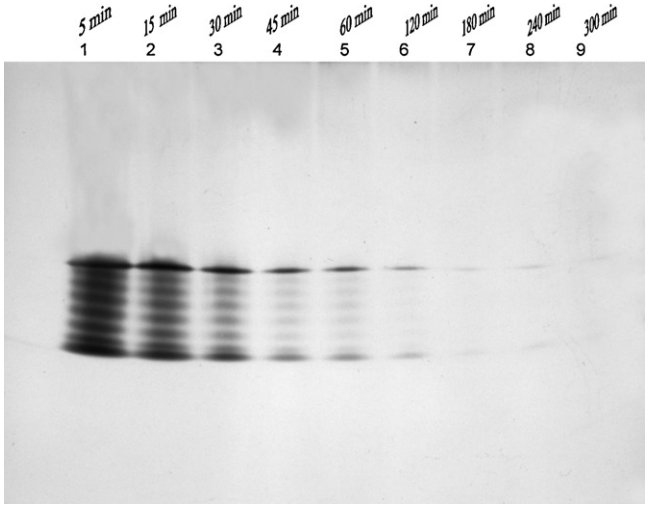

Fig. 5. PAGE analysis of HS203 in beagle dog plasma following i.v. administration of 60 mg/kg (from left to right: 5, 15, 30, 45, 60, 120, 180, 240, 300 min, respectively).

in this chromatograph, which is important for pharmacodynamic study of HS203 as a potential therapeutic agent for DMT2.

Silver staining PAGE analysis of HS203 (as shown in Fig. 5) showed that the maximum concentration of HS203 reached at 5 min after intravenous administration, which was consistent with that measured by HPLC-PC method. PAGE in combination with silver staining is a rapid and high sensitive method for the microanalysis of oligosaccharides (Ikegami-Kawai & Takahashi, 2002), and HS203 was monitored by PAGE in plasma until 120 min after administration. While the drug was detected by the HPLC-PC method until 5 h, indicating that the current HPLC-PC method is more sensitive than silver staining PAGE method to the analysis of HS203.

4. Conclusions

A rapid and sensitive HPLC-PC method was developed for the microdetermination of HS203 in beagle dog plasma and validated by a sensitive silver staining PAGE method. The chromatographic separation with an Asahipak GS-320 HQ column provided excellent resolution of HS203 and interfering compounds. The fluorometric postcolumn reaction with guanidine hydrochloride allowed highly sensitive and selective detection of HS203 in plasma samples and the LOD was 4 ng. This method was not only successfully applied to a pharmacokinetic study of HS203 in beagle dogs, but also used to monitor blood glucose level simultaneously after intravenous administration. These results indicated that the HPLC-PC method is very effective for pharmacokinetic and pharmacodynamic study of HS203 in blood plasma samples and also gave the potential for quantitative microdetermination of other oligosaccharide containing aldehyde group in biological materials.

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