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Determination of blood sugars by high pressure liquid chromatography with fluorescent detection[1]

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Abstract

In this study, a high pressure liquid chromatography method with fluorescent detector was developed to analyze blood galactose, lactose and glucose simultaneously. Plasma sugars were prepared as fluorescent derivatives to react with FMOC-hydrazine (9-fluorenyl methyl chloroformate). A C_{18} reversed phase column and a fluorescent detector were used and run in ambient. The resolution index of galactose and glucose derivatives in the analytical method was 1.15. The coefficients of variation of the analysis were less than 7.5%. The concentration of FMOC-hydrazine did not significantly influence the analytical results for determination of the concentration of galactose. However, the ratios of acetonitrile in the mobile phase significantly affected the analysis of the fluorescent derivatives of sugars. The sensitivity of this method for galactose detection was 5 μg ml^{-1}, and the required plasma volume for testing was only 25 μl each. This analytical method was successfully applied to study the pharmacokinetics of galactose in vivo in a rabbit model.

Keywords: Blood sugars; HPLC; Galactose

1. Introduction

Carbohydrate constitutes approximately 40% of the energy intake for neonates ingesting human milk or bovine milk. Lactose is the principle carbohydrate of mammalian milk. Galactose represents 50% of monosaccharide present in lactose, and is an important energy source in milk and an important metabolite of small intestinal digestion of lactose. After digestion and absorption, liver removes most of the dietary galactose on the first pass principally by converting it to glucose or glycogen [1–4]. Thus the rate of galactose clearance can be taken as an index for measurement of hepatic blood flow [5]. It appears that galactose does not directly stimulate insulin secretion [1,6–8]. Theoretically, it is possible that galactose administration parenterally could have advantages in terms of hepatic glucose output [7], control of hyperglycemia [9] and increased glycogen synthe-

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sis [4,5]. A precise and accurate method for quantitative analysis of lactose, galactose and glucose simultaneously is crucial for studying lactose absorption and galactose metabolism.

The earliest method to determine blood galactose included a fermentation technique to remove the blood glucose to measure the nonglucose-reducing substances [10,11]. Since the 1960s, standard methods have used the specific oxidative enzymes, galactose oxidase or lactose dehydrogenase, with indirectly coupled colorimetirc reactions to determine blood galactose [12–14]. Recently, high-performance liquid chromatography (HPLC) with pulsed amperometric detection has been applied to determine sugar alcohols [15,16]. The method is considered sensitive without chemical modification of sample sugars and sugar alcohols, however samples need to be purified before HPLC analysis in an ion-exchange column and to be eluted at a higher temperature (such as 80°C) for improving the resolution of monosaccharides. For increasing the sensitivity of detection, postcolumn derivatization has also been employed in HPLC methods for analysis of saccharides [17], and a number of fluorescent reagents have been examined [18–21]. FMOC-hydrazine has been synthesized as a precolumn fluorometric labeling reagent for reducing sugars such as glucose, galactose, mannose, fructose, fucose, ribose, xylose, arabinose, lactose, and maltose [22]. However, a similar method has not been used for plasma sugars detection. In this study, a sensitive method was established for determination of blood sugars. The reducing sugars were labeled with FMOC-hydrazine and an HPLC method with fluorescent detector was developed to analyze lactose, galactose and glucose simultaneously in the in vitro study. The analytical method was also applied to study the pharmacokinetics of galactose in vivo in a rabbit model.

2. Materials and methods

2.1. Materials

D-lactose monohydrate, D-glucose, D-galactose and D-xylose were ordered from E. Merck (Darmastadt, Germany). Hydrazine hydrate (Wako, Japan), 9-fluorenyl methyl chloroformate (FMOC) (Sigma, USA), acetonitrile (BDH, England), glacial acetic acid (E. Merck, Darmastadt, Germany) and alcohol (95%, Taiwan Tobacco and Wine Monopoly Bureau) were also used. Alcohol was redistilled before use. Other reagents were also used as reagent grade.

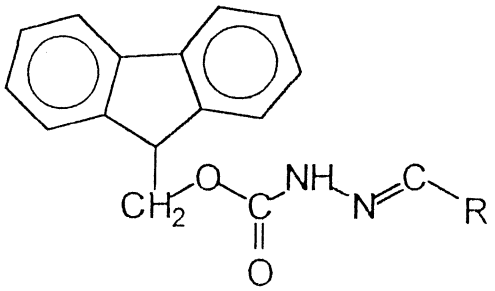

Fig. 1. FMOC-hydrazone sugar derivatives. R = lactose, galactose, glucose, and xylose.

2.2. Preparation of FMOC-hydrazine

The FMOC-hydrazine was prepared according to the method of Zhang et al. [23]. The procedure was briefly stated as follows. Hydrazine hydrate 10 ml was gradually added into the 250 ml of FMOC acetonitrile solution (1:250, w/v) which was stirred 30 min in room temperature, then vacuumed to dry. The crude product was recrys-

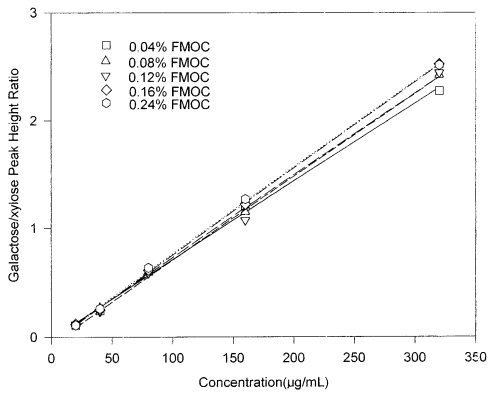

Fig. 2. The effects of various concentrations of FMOC-hydrazine on the determination of galactose fluorescent derivatives.

Table 1
Components of HPLC mobile phases (A–D) and washing eluant (E)

Mobile phases	0.5% acetic acid (%)	Acetonitrile (%)	Tetrahydrofuran (%)	Triethylamine (%)
A	76.6	23	0	0.4
B	73.6	26	0	0.4
C	71.6	28	0	0.4
D	70.6	28	1	0.4
E	64.6	2.5	0	0.4

tallized in acetonitrile. The purified white needle crystal of FMOC-hydrazine had a melting point 174–175°C.

The FMOC-hydrazine was dissolved in acetonitrile to obtain five different concentrations as 0.04, 0.08, 0.12, 0.16 and 0.24% (w/v) which were used to prepare sugar fluorescent derivatives.

2.3. Preparation of spiked plasma samples and internal standard

Galactose and lactose solutions were separately prepared in Milli-Q water at five different concentrations (200, 400, 800, 1600 and 3200 mg ml^{-1}). Then, rabbit plasma (0.8 ml) was added to various concentrations of lactose and galactose solutions, each 0.1 ml. The final preparations of spiked plasma samples contained galactose and lactose to be 20, 40, 80, 160 and 320 μg ml^{-1}.

Xylose was used as internal standard which was prepared in Milli-Q water as 1% (w/v) solution, then diluted with alcohol (fresh distilled) to 25 μg ml^{-1}.

2.4. Preparation of fluorescent derivatives of samples

The preparing method modified from the reported method for determining galactose or lactose [23]. Plasma samples including spiked plasma samples (25 μl) were added to 50 μl of xylose alcoholic solution as internal standard. After mixing, 0.8 ml of alcohol was added and mixed for another 1 min. Following this, the mixture was centrifuged at 1300*g* for 15 min. The supernatant 0.1 ml was added to 0.5 ml of 0.1% acetic acid alcoholic solution and FMOC-hydrazine solution (0.25 ml). Then, sugars and FMOC-hydrazine reacted in darkness at 65°C for 3 h. During the reacting period, samples were withdrawn and vortexed for 20 s at 30 min intervals. After finishing the reaction, a 0.2 ml aliquot was withdrawn, diluted with 0.8 ml of HPLC mobile phase, and analyzed by the HPLC method.

2.5. Pharmacokinetic study

New Zealand white rabbits, weighing 2.5–3 kg, either sex, were used in the study. Rabbits were anesthetized by intramuscular injection of ketamine (30 mg kg^{-1}) and xylazine (8 mg kg^{-1}). A catheter (PE-90, Clay Adams) was placed in the femoral vein and was connected to a three-way valve for collecting blood. Rabbits were intravenously injected galactose (5%, sterilized aqueous solution) with a dose 0.5 g kg^{-1} of body weight through the ear marginal vein. After administration of galactose, at times of 10, 20, 30, 40, 50, 60, 70, 80, 120 and 140 min 1 ml of blood was collected in a 5 ml vial (containing 50 units of heparin). Meanwhile, an equal volume of normal saline was injected to compensate the body fluid.

Blood samples were centrifuged at 1300*g* for 15 min, then plasma samples were obtained from the supernatant. Samples collected from time points at 10–60 min were further diluted 10 times before assay. Plasma levels of galactose were analyzed by a HPLC method after having been prepared as fluorescent derivatives.

2.6. HPLC conditions

In the study, a HPLC system was used in the analysis of sugar fluorescent derivatives. The ma-

Table 2
The HPLC analytical parameters for the determination of sugar fluorescent derivatives

Mobile phases	Capacity factor			R_s value of galactose and glucose
	Galactose	Glucose	Xylose	
A	9.43	10.22	15.31	1.5634
B	5.98	6.48	9.69	1.0167
C	4.36	4.88	8.15	1.1469
D	3.90	3.91	6.29	0.0220

$K' = (T_r - T_m)/T_m$.
$R_s = (T_{ry} - T_{rx})/(\frac{1}{2}W_x + \frac{1}{2}W_y)$.
K': capacity factor; T_r: retention time of sugar fluorescent derivatives; T_m: retention time of mobile phases; R_s: resolution; T_{ry}: retention time of galactose peak; T_{rx}: retention time of glucose peak; $\frac{1}{2}W_y$: half wave width of galactose peak; $\frac{1}{2}W_x$: half wave width of glucose peak.

jor part of the HPLC system was made by Jasco (Tokyo, Japan) which included two peristaltic pumps (880 PU), an autosampler (880 AS) with a loop injector (50 μl), a systemic controller (801 SC) to control the programming of mobile phases, a solvent mixer, a fluorescent detector (FP 210) and an integrator (SIC 12, Japan). A reversed-phase C_{18} column (3.2 × 150 mm, 5 μ, Inertsil, Vercopak) was used to analyze the fluorescent derivatives of sugars. The excitation and emission wavelengths of the fluorescent detector were 270 and 320 nm, respectively. During the HPLC analysis, injected samples were controlled at 5°C by a circulation water bath. Two mobile phases were used which composed of 0.5% acetic acid solution, acetonitrile and triethylamine with ratios of 716:280:4 and 646:250:4 (v/v). The flow rate of mobile phases was set at 0.5 ml min^{-1} and run in ambient. The analytical process included three steps, the first step 25 min with the first mobile phase, the second step 20 min with the second mobile phase for washing the column, the third step 45 min with the first mobile phase for returning the initial condition.

3. Results

3.1. Effects of FMOC-hydrazine concentrations

The FMOC-hydrazine reacted with glucose, xylose, lactose and galactose to form various sugars of FMOC-hydrazone derivatives. The structure is shown in Fig. 1. These derivatives had fluorescent chromophore to be detected by a fluorescent detector.

A serial concentration of FMOC-hydrazine did not significantly influence the analytical results for determination of the concentration of galactose (Fig. 2). For galactose concentration less than 80 μg ml^{-1}, the peak height ratios (galactose/xylose) were almost identical with no difference among the four levels of FMOC-hydrazine. For higher galactose concentrations, 160 and 320 μg ml^{-1}, there were little higher responses with increasing concentration of FMOC-hydrazine. However, using higher concentrations of FMOC-hydrazine, 0.16% and 0.24%, there was no difference in two levels. Therefore, we chose 0.24% FMOC-hydrazine in the preparation of the fluorescent derivatives of sugars.

3.2. Effects of mobile phases on HPLC analysis

The ratios of acetonitrile in the mobile phases (Table 1) affected the analysis of the fluorescent derivatives of sugars. Increasing the concentration of acetonitrile (23–28%) tended to decrease the retention times of sugar fluorescent derivatives. The HPLC analytical parameters of four different mobile phases are listed in Table 2. It seems that mobile phase A containing the lowest acetonitrile (23%) had the best resolution for galactose and glucose. Mobile phases B and C obtained about

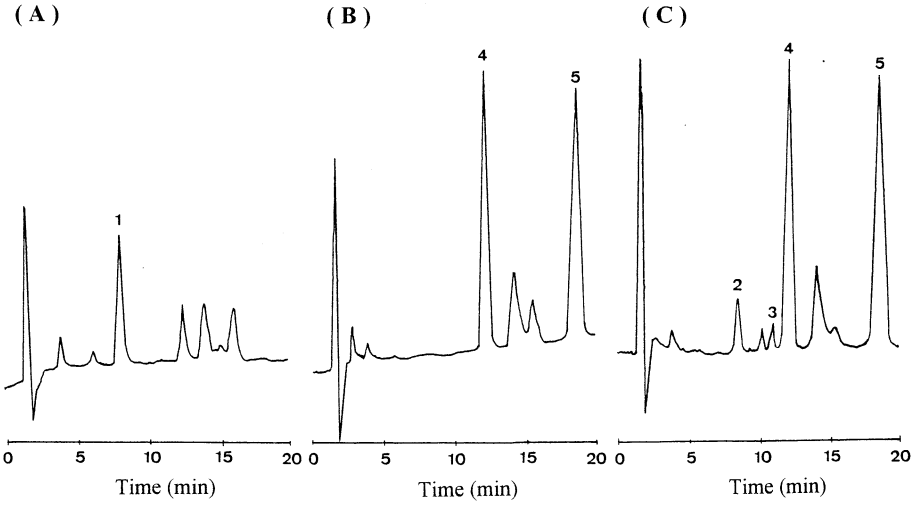

Fig. 3. Chromatograms of sugar fluorescent derivatives. Panel A: solution mixing with FMOC-hydrazine; Panel B: blank plasma without galactose and lactose; Panel C: spiked plasma sample containing galactose (20 μg ml^{-1}) and lactose (80 μg ml^{-1}). Peak 1: FMOC-hydrazine; Peak 2: lactose; Peak 3: galactose; Peak 4: glucose; Peak 5: xylose.

an equal resolution for galactose and glucose; these two mobile phases, especially mobile phase C, significantly reduced the retention time of xylose derivatives which was also the rate-limiting step for the analysis of sugar derivatives. Mobile phase D contained 1% of THF which did not improve the resolutions of the sugar derivatives. HPLC peaks of sugar (lactose, galactose and glucose) derivatives were overlapped in the system.

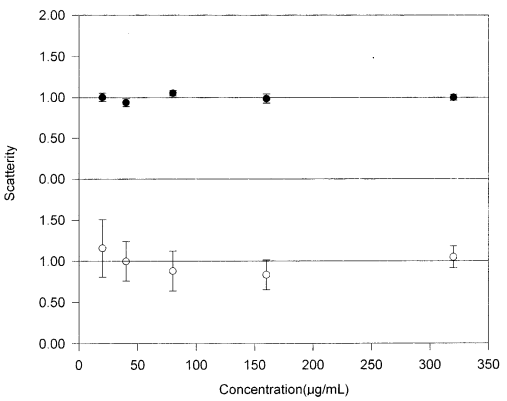

Fig. 4. The linearities of galactose (●) and lactose (○). Mean and standard deviation ($n = 3$).

3.3. Calibration curves

The spiked samples with galactose and lactose reacted with 0.24% FMOC-hydrazine which were analyzed by the developed HPLC method. The analytical process was divided three steps: the first step lasted 25 min, using mobile phase C; the second step lasted 20 min, using mobile phase E as washing eluant; and the third step lasted 40 min, using mobile phase C to return the initial condition. Fig. 3 shows the chromatograms of sugars. In panel A, 0.24% FMOC-hydrazine mixed with the mobile phase C, the major peak 1 was identified to be the FMOC-hydrazine. In panel B, blank plasma reacted with FMOC-hydrazine without adding galactose and lactose; endogenous glucose and internal standard were noticed in the chromatograms. In panel C, spiked plasma samples contained galactose and lactose which showed all four fluorescent derivatives of sugars.

The calibration curves of galactose and lactose were established for using the peak height ratios of two sugars and internal standard versus concentrations. Two regression lines ($Y = a \times X + b$; $Y =$ peak height ratio, $X =$ concentration) were obtained from 20 to 320 μg ml of the study; for

Table 3
Between-day variations of HPLC analysis for galactose fluorescent derivative[a]

Concentrations (μg ml^{-1})	Peak height ratio					Relative SD (%)
	Day 1	Day 2	Day 3	Mean[b]	SD	
20	0.1225	0.1207	0.1089	0.1175	0.0075	6.4
40	0.2692	0.2491	0.2491	0.2610	0.2595	3.9
80	0.6000	0.6139	0.6345	0.6161	0.0174	2.8
160	1.1473	1.2195	1.2651	1.2106	0.0594	4.9
320	2.4236	2.5262	2.5167	2.4889	0.5670	2.3

[a] Linear regression: $Y = 0.00773X - 0.03962$, $r = 0.9997$.
[b] $n = 3$.

galactose, $a = 0.00773 \pm 0.00010$, $b = -0.03962 \pm 0.02423$, $r = 0.9997$; for lactose $a = 0.00188 \pm 0.00012$, $b = -0.01466 \pm 0.03159$, $r = 0.9930$. The linearities of galactose and lactose are shown in Fig. 4 using a $(Y-b)/a$ versus X plot. The regression lines fit galactose well but only fairly well for lactose. Lactose is a disaccharide; the peak height of lactose fluorescent derivative is much less than that of monosaccharide in the analysis of HPLC with fluorescent detector [23]. That might be the reason we could not obtain good fitting for lactose. The lowest quantitative level of galactose was 5 μg ml^{-1} which was determined from spiked samples with coefficients of variation less than 7.5%. Between-day variations of galactose in the analytical method are shown in Table 3.

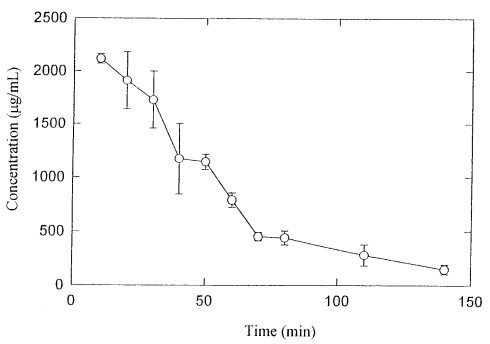

Fig. 5. The time course of plasma levels of galactose after intravenous administration of galactose (0.5 g kg^{-1}) in New Zealand White Rabbits. Mean and standard deviation ($n = 3$).

3.4. In vivo studies

The plasma levels of galactose in rabbits were determined by using the developed analytical method (Fig. 5). The time course of galactose in plasma fit one compartment model with intravenous bolus injection. The pharmacokinetic parameters are listed in Table 4. The half-life of galactose in rabbit was 23.3 min. The clearance and apparent volume of distribution of galactose were 4.13 ml min^{-1} kg^{-1} and 138.7 ml kg^{-1}, respectively.

4. Discussion

A sensitive HPLC method using a fluorescent detector was developed to determine blood sugar levels in this study. Although the FOMC-hydrazine method had previously been applied in the determination of monosaccharides in glycoproteins [23], it has never been applied to determine blood sugar levels. For the preparation of sugar fluorescent derivatives, 0.25 ml of various concentrations of FMOC-hydrazine (0.04–0.24%) were used to react with sugars and form a 1:1 derivative. Quantitative molecules of sugars in the highest concentration of spiked samples (i.e. a 25 μl plasma sample, spiked galactose and lactose each 320 μg ml^{-1}, also contained endogenous glucose 900 μg ml^{-1}; after deproteinization, a portion, 100/875, was used in the reaction) contained galactose (0.005 μmol), glucose (0.015 μmole), and lactose (0.0025 μmol); the total

Table 4
Phamacokinetic parameters in New Zealand White Rabbits after IV galactose (0.5 g kg^{-1})

	Rabbit 1	Rabbit 2	Rabbit 3	Mean	SD
K (min^{-1})	0.0337	0.0251	0.0319	0.0302	0.00454
Half life (min)	20.5	27.6	21.7	23.3	3.78
AUC (min. μg ml^{-1})	1.103×10^5	1.241×10^5	1.294×10^5	1.213×10^5	9.866×10^3
V_d [a] (ml kg^{-1})	138.0	161.4	115.7	138.7	22.82
Cl[b] (ml min^{-1} kg^{-1})	4.65	4.05	3.70	4.13	0.484

[a] V_d: apparent volume of distribution.
[b] Cl: clearance = dose/AUC.

molecules of sugars were about 0.025 μmol, the amount of FMOC-hydrazine (MW = 254.29) for 0.24% concentration (0.25 ml) was about 2.4 μmol; excess FMOC-hydrazine was used to complete the reaction. In this study, we found that 0.08 and 0.04% of FMOC-hydrazine obtained less peak ratio responses in 160 and 320 μg ml^{-1} of sugars in comparison with higher levels of FMOC-hydrazine.

For deproteinization, we used alcohol for removing the protein from the plasma. Although acetonitrile was a more efficient agent for deproteinization, sugars were found to be precipitated with protein for using acetonitrile in the deproteinization. Therefore, alcohol was used instead of acetonitrile in the study.

The ratios of acetonitrile in the mobile phase of the HPLC analysis dramatically affected the resolution of sugar fluorescent derivatives, slightly increasing the strength of acetonitrile, such as 23% to 28%, could be double the capacity factors of sugars. In the study with a mobile phase containing 28% acetonitrile, xylose fluorescent derivative was eluted at less than 20 min, but with a mobile phase with 23% acetonitrile, the derivative was eluted near 30 min. Glucose and galactose fluorescent derivatives could be separated in all three strengths of acetonitrile without THF. To reduce the analytical time, 28% acetonitrile was used in the study. THF has been used to minimize the tailing of the peak in the HPLC analysis. However, we found that this was of no benefit in the study. It may be due to two monosaccharide derivatives only slightly different in stereo structure; the lipophilic properties were about equal. Thus, no improvement was noticed for the mobile phase with THF.

In the study, the lowest analyzed level of galactose in plasma was 5 μg ml^{-1}, which is sensitive enough for the investigation of pharmacokinetics of galactose. Although HPLC with pulsed amperometric detection offers a direct determination with a detected limit of 10 pmol (1.8 ng or 10 μl injection with a concentration of 0.18 μg ml^{-1}), samples need to be prepared with more steps to separate from other interfering biological constituents before HPLC analysis. In addition, samples need to be separated by ion-exchange column with a higher temperature for resolving sugars [15]. Our method offers a sensitive and accurate way for quantitative determination of plasma galactose. In the pharmacokinetic study in rabbits we could not detect lactose in the plasma, while the glucose levels in plasma were found to be almost always around 800–1000 μg ml^{-1}.

Sugar fluorescent derivatives were unstable in room temperature and were light sensitive. The degradation half-life was approximate 12 h. Thus, it was necessary to store these samples under 5°C. In this condition, samples of fluorescent derivatives could be kept more than one month. This is consistent with other reports [23].

For further application of this newly developed method it would be very useful for studying galactose metabolism in neonates because of its minimal sample volume requirement. This method might be helpful to settle the controversy about whether premature and full term infants could metabolise galactose normally at birth [24,25]. It could also be a powerful method to explore the

advantages and safety for galactose nutrient intravenous administration.

5. Conclusions

In this study a HPLC method combining a fluorescent detector was developed to determine sugar fluorescent derivatives including lactose, galactose, glucose and xylose. These sugars could be measured simultaneously after preparation as fluorescent derivatives. The concentration of FMOC-hydrazine did not significantly influence the analytical results for determination of the concentration of galactose. The ratios of acetonitrile in the mobile phase affected the analysis of the fluorescent derivatives of sugars. The method was successfully applied in the determination of pharmacokinetics of galactose after IV administration in rabbits. In vivo studies showed that the time course of galactose in plasma fit one compartment model with intravenous injection. The required plasma sample volume for testing is only 25 μl. The sensitivity of this method for plasma galactose detection was 5 μg ml^{-1}. This method could be further applied to clinical tests for monitoring galactose metabolism in neonates.

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