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Fluorimetric determination of D-lactate in urine of normal and diabetic rats by column-switching high-performance liquid chromatography

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Abstract

A highly sensitive method for the fluorimetric determination of D-lactate in urine of normal and diabetic rats was developed using column-switching high-performance liquid chromatography (HPLC) with an octadecylsilica (ODS) column connected to a chiral column, an amylose tris(3,5-dimethylphenylcarbamate) coated on silica gel (Chiralpak AD-RH). During the separation step on the ODS column, the peak fraction of the (D+L)-lactate derivative with a fluorescence reagent, 4-nitro-7-piperazino-2,1,3-benzoxadiazole (NBD-PZ), was introduced directly to the chiral column by changing the flow of the eluent via a six-port valve. The D-lactate derivative was separated enantiomerically from the L-lactate derivative, and the enantiomeric ratio was determined from the chromatogram. The accuracy values for the determination of D-lactate in 20 μL of rat urine were 96.93–104.85%, and the intra- and inter-day precision values were within 0.80 and 14.44%, respectively. The detection limit for D-lactate was approximately 10 nM (with a signal-to-noise ratio of 3).

The proposed HPLC method was applied to the urine of normal and diabetic rats induced by intraperitoneal administration of streptozotocin, and significant increases in D-lactate excreted into the urine were observed in diabetic rats compared to normal rats. In diabetic rats, D-lactate concentrations showed a rising tendency from the seventh day and then remained stable from the 28th day after induction, suggesting that urinary D-lactate may be used as an indicator to determine the diabetic stage and the level of kidney damage.

Keywords: D-Lactate; Column-switching HPLC; Enantiomeric separation; NBD-PZ; Fluorescence derivatization; Chiralpak AD-RH

1. Introduction

Although D-lactate exists in trace amounts compared with L-lactate in mammals, the D-lactate level has been considered an indicator of some human diseases, such as diabetes [1–3], encephalopathy [4], D-lactate acidosis [3–5], and appendicitis [6], in which plasma D-lactate concentrations are increased. Therefore, measurement of D-lactate in clinical samples is valuable.

There are a variety of methods for determining D-lactate in biological samples. One widely used method utilizes D-lactate dehydrogenase (D-LDH) [1,2,7–9], which catalyzes the conversion of D-lactate to pyruvate in the presence of nicotinamide adenine dinucleotide (NAD^+) as a co-factor, and the absorption of the resultant NADH formed from NAD^+ is measured [9] or it is determined fluorimetrically [1]. Although this method can rapidly determine D-lactate, the enzymatic reaction tends to be influenced by a number of endogenous compounds, such as fructose 1,6-bisphosphate, 3-

Abbreviations: NBD-PZ4-nitro-7-piperazino-2,1,3-benzoxadiazole; DBD-PZ4-(*N*,*N*-dimethylaminosulfonyl)-7-piperazino-2,1,3-benzoxadiazole; TPPtriphenylphosphine; DPDS,2,2′-dipyridyl disulfide; TFAtrifluoroacetic acid

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phosphoglyceric acid, pyruvate, L-lactate, and *S*-lactonyl glutathione [9,10], which can affect the accuracy and precision of this method.

To avoid the problems mentioned above, HPLC methods with enantiomeric separation of D- and L-lactate by a fluorescence detector following precolumn fluorescence derivatization were proposed in our previous study [11–14]. The sensitivity of these methods is better than other HPLC methods using a UV detector; and it is adequate for detecting trace amounts of D-lactate in biological samples.

D-Lactate has one carboxyl group and one hydroxyl group in its structure, and either group can be a target as a derivatization site with a fluorogenic reagent. Acyl chloride-type reagents are often utilized for the derivatization of the hydroxyl group, but this kind of reagent reacts readily with water to produce the hydrolysis product [11,15]. Therefore, the acyl chloride-type fluorogenic reagent is unsuitable to derivatize D-lactate in biological samples which contain water. From this reason, the carboxyl group of D-lactate was chosen as the derivatization site. In this paper, we investigated the enantiomeric separation of D- and L-lactate derivatized with two reagents, 4-nitro-7-piperazino-2,1,3-benzoxadiazole (NBD-PZ) or 4-(*N*,*N*-dimethylaminosulfonyl)-7-piperazino-2,1,3-benzoxadiazole (DBD-PZ) as the pre-column fluorogenic reagent (Fig. 1). The reaction of the enantiomers of lactate with the derivatization reagents is carried out in mild reaction conditions at room temperature within a short time. In addition, their excitation and emission were at long wavelengths, which provides a distinct advantage in biological samples because there is little interference from endogenous compounds [13,16]. Then, the separation of D- and L-lactate derivatives was optimized on the following polysaccharide-type chiral stationary phases, cellulose tris(3,5-dimethylphenylcarbamate) coated on silica gel (Chiralcel OD-RH), cellulose tris(4-methylbenzoate) coated on silica gel (Chiralcel OJ-R), and amylose tris(3,5-dimethylphenylcarbamate) coated on silica gel (Chiralpak AD-RH) (Fig. 2). Among the three types of columns, only the Chiralpak AD-RH column produced satisfactory separation.

In our previous study, using column-switching HPLC including octylsilica and Chiralpak AD-RH columns, a higher D-lactate concentration in the serum of diabetic patients compared to normal subjects was observed [17]. This result supports the fact that the increased D-lactate level in plasma might be one of the clinical markers for diabetes. Among diabetic complications, diabetic nephropathies are very serious and irreversible, and ultimately can cause death. Considering this, early diagnosis for prevention of diabetic nephropathies can reduce the morbidity and mortality of diabetic patients. Monitoring markers for nephropathies, especially in the urine, is crucial to understanding progress of diabetic complications.

Thus, in the present study, urinary D-lactate concentrations in normal rats and those with streptozotocin-induced diabetes were determined by the proposed HPLC method, and alterations of D-lactate levels were pre-clinically investigated in relation to kidney damage with diabetes.

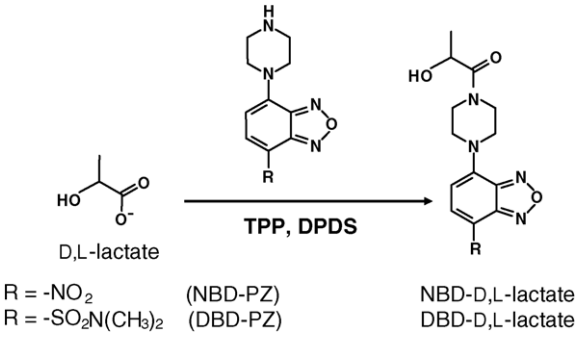

Fig. 1. Derivatization scheme of lactic acid with NBD-PZ or DBD-PZ.

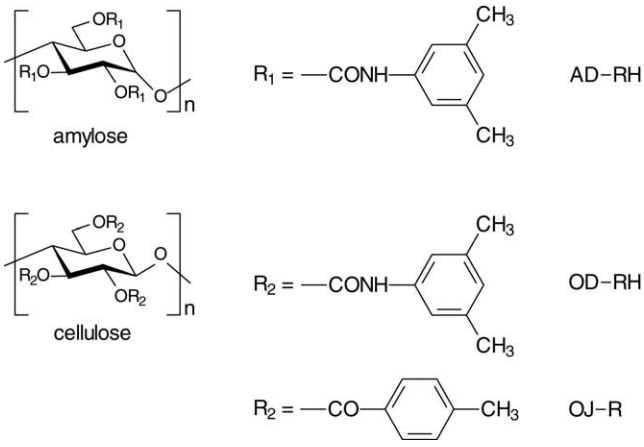

Fig. 2. Structures of the chiral moiety in the Chiralcel OD-RH, Chiralcel OJ-R, and Chiralpak AD-RH columns.

2. Materials and methods

2.1. Chemicals

Lithium D- and L-lactate, streptozotocin, creatinine, cimetidine, and sodium lauryl sulfate were purchased from Sigma Chemical (St. Louis, MO, USA). NBD- and DBD-PZ, triphenylphosphine (TPP) and 2,2′-dipyridyl disulfide (DPDS) were from Tokyo Kasei Chemicals (Tokyo, Japan). Trifluoroacetic acid (TFA), propionic acid, citric acid, sodium hydroxide, and hydrochloric acid were from Nacalai Tesque (Tokyo, Japan). Sodium dihydrogen phosphate and *o*-phosphoric acid were obtained from Riedel-de Haën (Seelze, Germany), and acetone was from Alps Chemical (Taipei, Taiwan). Methanol (MeOH) and acetonitrile (CH_3CN) were of HPLC grade from Merck (Darmstadt, Germany).

2.2. Animal experiment

Sprague–Dawley male rats (National Laboratory Animal Breeding and Research Center, Taipei, Taiwan) were kept in

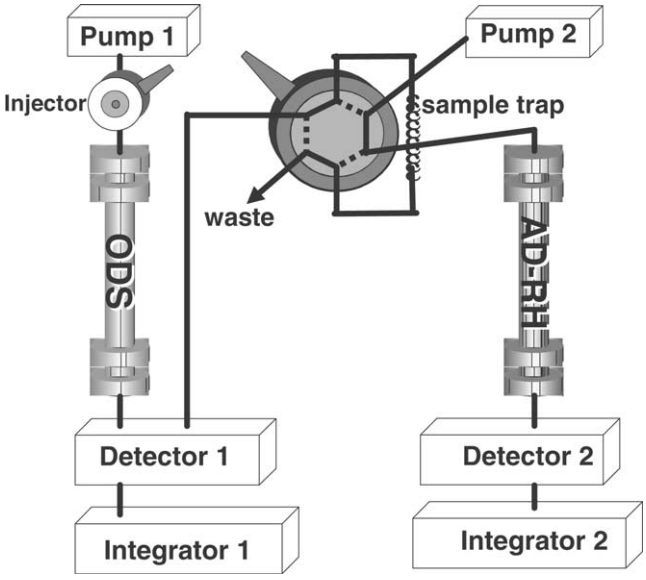

Fig. 3. The column-switching HPLC system. The six-port valve positions A and B are represented by the solid and dotted lines, respectively. A TSKgel ODS-80Ts column was used as the ODS column, while OD-RH, OJ-R, and AD-RH columns were used for the chiral column. For analysis of D-lactate in rat urine, the mobile phase for pump 1 was CH_3CN:MeOH:H_2O (10:20:70) at a flow rate of 0.7 mL/min, and MeCN:H_2O (60:40) for pump 2 at a flow rate of 0.3 mL/min. Both detectors 1 and 2 were set to 547 nm for fluorimetric detection with 491 nm as the excitation wavelength.

an environmentally controlled room with free access to water and food at least 1 week prior to use. To induce diabetic rats, streptozotocin (STZ) was dissolved in 50 mM citrate buffer (pH 4.5) and immediately administered intraperitoneally at a dose of 80 mg/kg. As a control, 50 mM citrate buffer was administered under the same conditions.

2.3. HPLC apparatus

A block diagram of the HPLC apparatus used in this study is shown in Fig. 3.

The HPLC equipment used in this study consisted of a model 7000, type 70 switching valve with a 100 μL loop as a sample trap (Rheodyne, Rohnert Park, CA, USA), two L-7100 intelligent pumps (Hitachi, Tokyo, Japan), an injection valve with a 20 μL sample loop, F-1000 and L-7485 fluorescence detectors (Hitachi), and two D-2500 chromato-integrators (Hitachi). The Chiralcel OD-RH (15 cm × 0.46 cm ID, particle size 5 μm), Chiralcel OJ-R (15 cm × 0.46 cm ID, particle size 10 μm), and Chiralpak AD-RH (15 cm × 0.46 cm ID, particle size 5 μm) columns were obtained from Daicel (Osaka, Japan). TSKgel ODS-80Ts (15 cm × 0.46 cm ID, particle size 5 μm) column was purchased from Tosoh (Tokyo, Japan).

2.4. Derivatization procedure

D- and L-Lactate derivatized with NBD-PZ proceeded as follows: 20 μL of 10 mM D- or L-lactate dissolved in H_2O was added to 50 μL of 10 mM NBD-PZ in CH_3CN in the presence of 50 μL each of 280 mM TPP and DPDS in CH_3CN. After standing for 2 h at room temperature, 330 μL of 0.1% TFA in H_2O was added to terminate the reaction. In order to remove the excess NBD-PZ, 100 μL of the resultant solution was loaded onto a mobile phase preconditioned solid-phase extraction cartridge, Empore^{TM} SBD-RPS (4 mm/1 mL), for complete elution, another 100 μL of the mobile phase was loaded, then the elute solutions were combined. An amount of 20 μL of the elute solution was injected into the HPLC.

D- and L-Lactate derivatized with DBD-PZ were prepared in the same manner as that with NBD-PZ except for the following changes in concentrations and volumes of reagents: 10 μL of 2 mM D- and L-lactate, 50 μL of 20 mM DBD-PZ, 50 μL each of 140 mM TPP and DPDS, and 640 μL of 0.1% TFA in a solution of H_2O/CH_3CN (75/25, v/v). An amount of 100 μL of the resultant solution was loaded onto a cation exchange cartridge, PL-SCX (Polymer Laboratories, MA, USA), which was preconditioned with 200 μL of a solution of H_2O/CH_3CN (40/60), to remove the excess DBD-PZ. The eluent was filtered, and another 100 μL of the solution mentioned above was loaded, then 5 μL of the combined filtrate was injected into the HPLC.

2.5. Enantiomeric separation on OD-RH, OJ-R, and AD-RH columns

The enantiomeric separation of D- and L-lactate derivatives was investigated using OD-RH, OJ-R, and AD-RH columns. To search for an adequate method, CH_3CN, MeOH, or mixtures of them with water in different ratios were used as the mobile phases (compositions are listed in Table 1A–C). The chiral column efficiency was expressed as the capacity factor (k), separation factor (α), and the resolution (Rs). To optimize the enantioseparation of D- and L-lactate, the effect of the flow rate was also investigated with the AD-RH chiral column. A solution composed of CH_3CN/H_2O (60/40) was used as the mobile phase, and an adequate flow rate of 0.3 mL/min was selected.

Fluorimetric detection was performed at 547 nm with an excitation wavelength of 491 nm for NBD-PZ and at 560 nm with 450 nm for DBD-PZ, respectively.

2.6. Analysis of D-lactate in rat urine

Urine samples were collected from normal or diabetic rats at 0, 3, 7, 14, 21, 28, 42 and 56 days after administration of streptozotocin. An amount of 20 μL of rat urine was added to 10 μL of 1 mM propionic acid in water as the internal standard and then vigorously mixed with 170 μL of CH_3CN for deproteinization. The solution was centrifuged at 700 × g for 5 min, and 100 μL of the supernatant was added to 100 μL of 8 mM NBD-PZ to perform the derivatization as described above. After elution from the SPE cartridge, 20 μL of the elute solution was injected into the HPLC. The mobile phase for the ODS separation was as follows: $CH_3CN/MeOH/H_2O$

Table 1
Effect of mobile phase compositions on capacity factors (k_D, k_L), the separation factor (α), and resolution (Rs) of the D,L-lactate derivative with NBD- or DBD-PZ on Chiralcel OD-RH (A), Chiralcel OJ-R (B), and Chiralpak AD-RH chiral stationary-phase (C) columns

Mobile phase composition	NBD-PZ				DBD-PZ			
	k_D	k_L	α	Rs	k_D	k_L	α	Rs
(A)								
MeCN:H_2O								
20:80	15.42	16.19	1.05	1.06	n.d.	n.d.	n.d.	n.d.
40:60	1.64	1.64	1	0	1.49	1.49	1	0
60:40	0.59	0.59	1	0	0.42	0.42	1	0
80:20	0.30	0.30	1	0	0.22	0.22	1	0
MeOH:H_2O								
40:60	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
60:40	n.d.	n.d.	n.d.	n.d.	11.70	11.70	1	0
80:20	5.33	5.59	1.05	0.43	2.67	2.67	1	0
MeCN:MeOH:H_2O								
30:20:50	2.51	2.51	1	0	2.16	2.16	1	0
75:20:05	0.31	0.31	1	0	0.19	0.19	1	0
(B)								
MeCN:H_2O								
20:80	4.06	4.06	1	0	7.17	9.45	1.32	5.40
40:60	0.46	0.46	1	0	0.49	0.56	1.15	0.55
60:40	0.11	0.11	1	0	0.14	0.14	1	0
80:20	0.10	0.10	1	0	0.08	0.08	1	0
MeOH:H_2O								
60:40	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
80:20	2.26	2.26	1	0	1.97	1.97	1	0
MeCN:MeOH:H_2O								
30:20:50	0.67	0.67	1	0	0.67	0.82	1.21	0.76
75:20:05	0.19	0.19	1	0	0.09	0.09	1	0
(C)								
MeCN:H_2O								
20:80	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
40:60	3.29	4.61	1.40	3.07	2.52	2.52	1	0
60:40	1.19	1.70	1.43	2.63	0.75	0.75	1	0
80:20	0.80	1.14	1.43	1.83	0.44	0.44	1	0

n.d., not determined.

(10/20/70) was isocratically eluted from 0 to 70 min at a flow rate of 0.7 mL/min, then 100% CH_3CN from 70 to 85 min at 0.7 mL/min for washing the ODS column.

2.7. Determination of creatinine in urine

A method previously described by Marsilio et al. [18] was used with some modifications. Briefly, 100 μL of 2.5- to 40-fold-diluted urine with water was added to 20 μL of 2 mM cimetidine in a 10 mM HCl aqueous solution as the internal standard, and then the resultant mixture was added to 400 μL of acetone. Samples were vortex-mixed for 1 min and then centrifuged at 5500 × *g* for 3 min. An amount of 300 μL of the supernatant was evaporated to dryness under reduced pressure, and this was reconstituted with 100 μL of the mobile phase. After centrifugation at 5500 g for 1 min, 20 μL of the supernatant was analyzed by HPLC using a Capcell Pak C_{18} column (25 cm × 0.46 cm ID, particle size 5 μm, Shiseido, Tokyo, Japan) with an L-4200 UV–vis detector (Hitachi, Tokyo, Japan) monitored at 234 nm. The mobile phase consisted of acetonitrile and 100 mM of a sodium dihydrogen phosphate buffer solution containing 30 mM sodium lauryl sulfate adjusted to pH 3.0 with *o*-phosphoric acid (36/60, v/v). The flow rate was at 0.8 mL/min.

3. Results and discussion

3.1. Enantiomeric separation of D- and L-lactate derivatives

In our previous study, we employed NBD-PZ and a Chiralpak AD-RH column as the fluorescence reagent and chiral column for the enantiomeric separation of D,L-lactate [17]. In the present study, using other polysaccharide-type chiral columns, Chiralcel OD-RH and OJ-R, as well as another fluorescence reagent, DBD-PZ, the enantiomeric separation of D,L-lactate was precisely investigated.

The *k*, α, and Rs values of NBD-PZ or DBD-PZ-D,L-lactate on the Chiralcel OD-RH, Chiralcel OJ-R, and Chiralpak AD-RH columns are summarized in Table 1A–C, respectively. The OD-RH column produced partial resolution

of NBD-PZ-D- and L-lactate when using the mobile phases containing 20% CH_3CN in water, but the retention times were too long (108 min for D-lactate and 113 min for L-lactate). When the mixture of MeOH with water was used as the mobile phase, NBD-PZ-D- and L-lactate were not eluted from the column until 75% MeOH in the mobile phase was used (data not shown). In the case of DBD-PZ-D- and L-lactate, the derivatives were not resolved at all on the Chiralcel OD-RH column using any of the test compositions of CH_3CN, MeOH, and their mixtures with water as the mobile phases.

As indicated in Table 1B, NBD-PZ-D- and L-lactate were not separated on the Chiralcel OJ-R column with any of the test mobile phase compositions. NBD-PZ-D- and L-lactate were retained on the column when using MeOH comprising less than 80% of the mobile phase. This phenomenon was also observed for DBD-PZ derivatives. A partial resolution of DBD-PZ-D,L-lactate was achieved on the Chiralcel OJ-R column using less than 40% acetonitrile in water as the mobile phase. While the enantiomeric resolution could be improved by increasing the content of water in the mobile phase, the peaks of DBD-PZ-D- and L-lactate became broad, and peak tailing also occurred. The resolution of the enantiomers reached 1.50 when the mobile phase contained 60% water (H_2O:MeOH:MeCN = 60:10:30, data not shown), but the resolution was not sufficient to separate the enantiomers in biological samples, which contained much more L-lactate than D-lactate.

Because neither NBD- nor DBD-PZ-D,L-lactate was eluted when the Chiralpak AD-RH column was used with methanol in the mobile phase, we solely evaluated the separations of D,L-lactate derivatives on the AD-RH column with different percentages of acetonitrile in water as the mobile phase. As shown in Table 1(C), there was no resolution of DBD-PZ-D-lactate or L-lactate on the Chiralpak AD-RH column; however, an ideal separation of NBD-PZ-D-lactate and L-lactate derivatives was achieved on the Chiralpak AD-RH column for the same mobile phase condition. The peaks of NBD-PZ-D,L-lactate were sharp when the mobile phase contained more than 60% acetonitrile. The Chiralpak AD-RH column using MeCN/H_2O (60/40) as the mobile phase was the best condition for resolving the enantiomers of lactate derivatized with NBD-PZ.

As summarized in Table 1(A–C), the Chiralpak AD-RH column seemed to possess the best resolution capacity among these three chiral columns. This may be attributed to the fact that the amylose-based chiral stationary phase has a helical structure giving well-defined grooves different from the corresponding cellulose analogues, which appear to be more linear and rigid in nature. Hence, the Chiralpak AD-RH column can provide a greater chiral environment for lactate enantiomers [19,20].

In addition, for achieving enantiomeric separation, the occurrence of interactions, including hydrogen bonding and π–π interactions, between enantiomers and the stationary phases should be a requisite. The carbonyl oxygen molecules on the D- and L-lactate derivatives might form hydrogen bonds with the NH protons on the carbamate moiety; another hydrogen bonding might be formed between the carbamate oxygen and the hydroxyl groups at the chiral carbon of D- and L-lactate derivatives (Fig. 2). Because 4-methyl benzoate is the substituent on the ester moiety in the Chiralcel OJ-R column, there is no NH proton on the OJ-R column for forming hydrogen bonds with the hydroxyl groups of NBD-PZ-D,L-lactate. In addition, the presence of the two methyl groups of 3,5-dimethylphenyl carbamate moieties on the Chiralcel OD-RH and Chiralpak AD-RH columns increases the π basicity of the phenyl moieties, which results in greater magnitudes of π–π interactions in comparison to the Chiralcel OJ-R column, which has only one electron-donating methyl group on the benzene ring. Therefore, 3,5-dimethylphenyl carbamate is considered to be a more suitable substituent to resolve NBD-PZ-D,L-lactate than 4-methyl benzoate in the stationary phase [15,20]. The phenomenon of the protic solvent, methanol, in the mobile phase being unable to separate the enantiomers may be explained by methanol's ability to intercept the hydrogen bonding interactions resulting in poor resolution [15].

On the other hand, we previously tried to use the AD-RH column and MeCN/H_2O as the mobile phase for separating D- and L-3-hydroxybutyrate (3-HB) derivatized with NBD-PZ, but satisfactory resolution was not obtained [21]. Chemically, D- and L-3-hydroxybutyrate have just one more methylene group in the alkyl chain than D- and L-lactate. The phenomenon might have been due to the poor interactions caused by repulsive force between the alkyl chains inside the AD-RH column and the longer alkyl chains of D- and L-3-hydroxybutyrate derivatives, and/or the lower rigidity of D- and L-3-hydroxybutyrate than D- and L-lactate. Therefore, insufficient resolution of D- and L-3-hydroxybutyrate derivatives using AD-RH was apparent, since conformational rigidity has been reported to be important for the enantiomer resolution under reversed-phase conditions [21,22].

Finally, we concluded that the use of NBD-PZ and the Chiralpak AD-RH column as the fluorescence reagent and chiral column, respectively, gave the best results for the enantiomeric separation of D,L-lactate.

3.2. Application to rat urine

The proposed HPLC method was applied to determine D-lactate levels in the urine of normal and diabetic rats. Because much-greater amounts of total urine are excreted in the diabetic stage than in a normal situation, this dilutes the D-lactate concentration in the urine, and thus a highly sensitive method for the determination of urine D-lactate should be useful.

(D + L)-Lactate in normal and diabetic rat urine was derivatized with NBD-PZ, separated on the ODS column, and determined fluorimetrically (Fig. 4A and B). Using the proposed HPLC method, 20 μL of urine volume was sufficient for D-lactate determination. The calibration curve, which was constructed by plotting the concentrations of D- and L-lactate versus the peak area ratio (D- and L-lactate/internal stan-

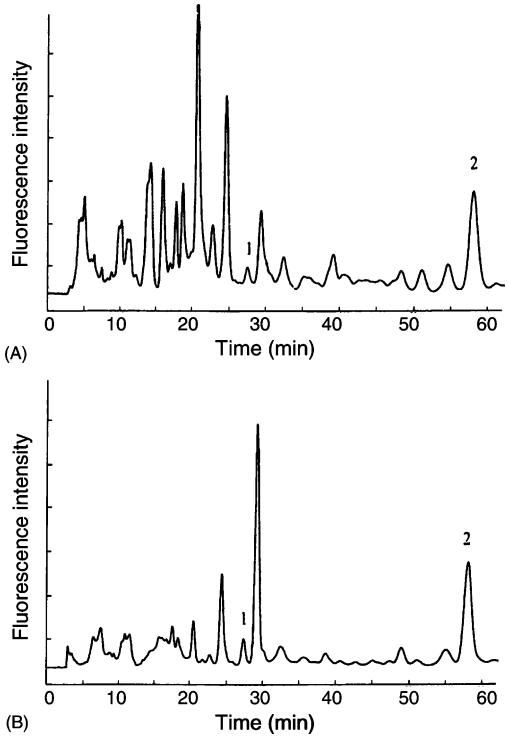

Fig. 4. Chromatograms of samples from the urine of normal (A) and diabetic rats (B) derivatized with NBD-PZ separated on the ODS column. (1) Lactate derivative; (2) propionate derivative (I.S.).

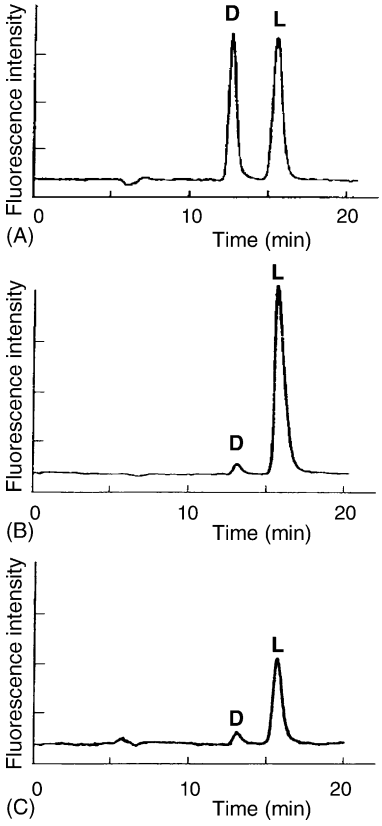

Fig. 5. Chromatograms of resolved enantiomers of the standard D,L-lactate (A), urine of normal rats (B) and diabetic rats (C) derivatized with NBD-PZ on a Chiralpak AD-RH column using a mobile phase of CH_3CN:H_2O (60:40).

dard), showed good linearity, and had a correlation coefficient of 0.998. The peak fraction of the (D + L)-lactate derivative, which was separated on the ODS column, was directly introduced to the Chiralpak AD-RH column via a six-port valve. Then, the D- and L-lactate derivatives were enantiomerically separated, and the enantiomeric ratio was determined from the chromatogram (Fig. 5). The intra- and inter-day precision values were within 0.80–14.44%. The accuracy values for the determination of D-lactate in urine were 96.93–104.85%. The detection limit for D-lactate was approximately 10 nM (with a signal-to-noise ratio of 3). Since diabetic rats excrete much greater volumes of urine than normal subjects, the urinary concentration of D-lactate in diabetic rats is inevitably diluted. Therefore, when comparing D-lactate levels between normal and diabetic rats, we employed a urinary compound, creatinine, to correct for the excreted urine volume, because urinary creatinine levels have commonly been used for assessing the glomerular filtration rate (GFR). Hence, D-lactate excretion in urine was expressed as a ratio of urinary creatinine (mmol/mol creatinine) [23]. Fig. 6 shows the mean ± S.D. of the D-lactate/creatinine ratio in the urine of normal and diabetic rats, and the ratios of D-lactate/creatinine of diabetic rats significantly increased compared to those of normal rats. After receiving STZ for 1 month, significant kidney hypertrophy was observed in the diabetic rats (5.2 ± 0.4 g versus 3.4 ± 0.1 g in the normal group, $P < 0.1$). Ward et al. reported that the GFR, urine flow rate, and urinary calcium were significantly increased on 7th day in rats with streptozotocin-induced diabetes [24]. These data suggest that drastic physiological and morphological changes occur in the kidney of diabetic rats. As shown in Fig. 6, urinary D-lactate concentrations showed a rising tendency from 7th day and then remained stable from the 28th day after diabetes was induced. These phenomena observed in Fig. 6 are coincident with a report by Kondoh et al., who determined D-lactate by their enzymatic method and showed that much-greater amounts of D-lactate were excreted in the urine of diabetic and fasted rats [25]. Considering these results together, urinary D-lactate may be used as an indicator to screen for early stages of kid-

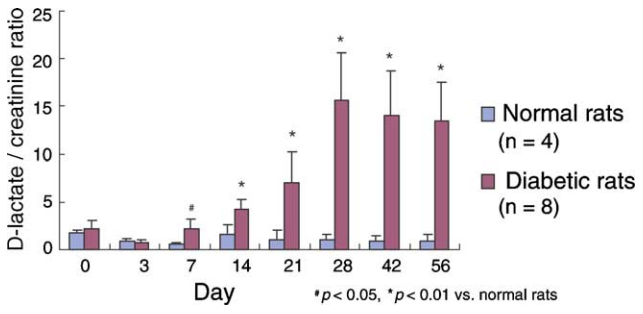

Fig. 6. Ratio of D-lactate (μM) to creatinine (mM) in the urine of normal and diabetic rats. $^{\#}P < 0.05$, $^{*}P < 0.01$ vs. normal rats.

ney injury, and the determination of urinary D-lactate may help us to understand the pathological conditions of kidneys of patients suffering from these diseases.

4. Conclusions

We have developed an HPLC method for the highly sensitive determination of D-lactate in 20 μL of rat urine, and found that urinary D-lactate was drastically increased in the diabetic stage induced by streptozotocin. With this proposed HPLC method, urinary D-lactate in diabetic patients and normal subjects will also be investigated.

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