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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.

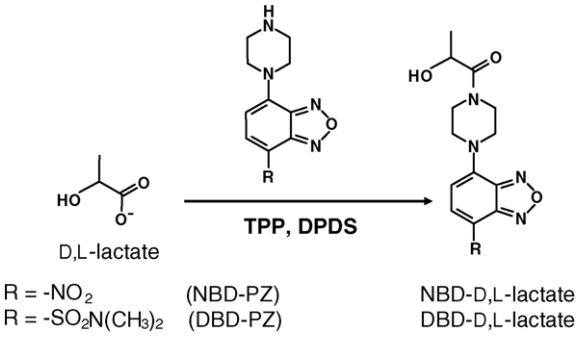

Fig. 1. Derivatization scheme of lactic acid with NBD-PZ or DBD-PZ.

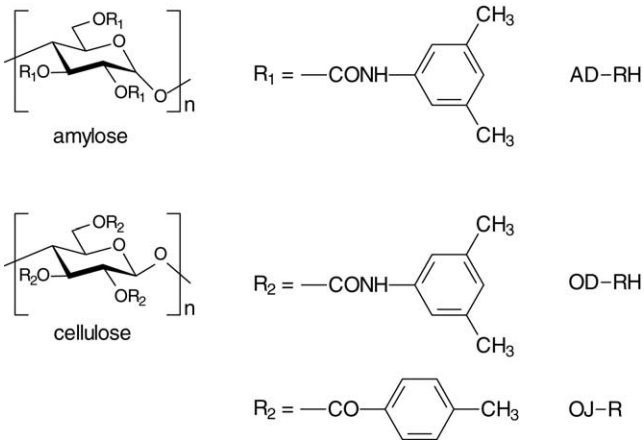

Fig. 2. Structures of the chiral moiety in the Chiralcel OD-RH, Chiralcel OJ-R, and Chiralpak AD-RH columns.

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