附件：封面格式

行政院國家科學委員會補助專題研究計畫成果報告

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※生物體內 D-, L-乳酸及 D-, L-3-羥基丁酸鏡像異構物之高效能液相層析分析法的開發※
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計畫主持人：李仁愛

共同主持人：

計畫參與人員：蔡屹喬、王志淳、廖紫歆

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行政院國家科學委員會專題研究計畫成果報告

生物體內 D-, L-乳酸及 D-, L-3-羥基丁酸鏡像異構物之高效能液相層析分析法的開發

Enantiomeric determination of D-,L-lactate and D-,L-3-hydroxybutyrate in biological samples using a column-switching HPLC

計畫編號：NSC 90-2320-B-038-029-

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主持人：李仁愛　台北醫學大學藥學系

計畫參與人員：蔡屹喬、王志淳、廖紫歆　台北醫學大學藥學系

一、中文摘要

乳酸具有 D-與 L-型之鏡像異構物。動物體內之 L-乳酸是較主要的代謝產物，而 D-乳酸則僅是微量物質。但 D-乳酸會在某些情形下(如糖尿病) 造成酸中毒。然而目前 D-乳酸仍缺乏簡便且準確的分析方法，本研究建立一套分析系統，使用管柱切換之高效能液相層析儀，搭配螢光偵測法，以掌性管柱 Chiralpak AD-RH 分析檢品中被 NBD-PZ 衍生化試劑衍生的 D-乳酸的含量，其準確度為 96.93 – 104.85 %，同日間與異日間之精密度介於 0.80 至 14.44 %。將此分析方法應用於糖尿病老鼠之尿液的分析，發現 D-乳酸含量在糖尿病老鼠之尿液中明顯地上升。可供進一步探討這些異構物間的組成比例與含量和糖尿病的併發症，如酸中毒或腎功能障礙間之關連性。

關鍵詞：D-乳酸，NBD-PZ 衍生化試劑，掌性管柱 Chiralpak AD-RH，螢光偵測法

Abstract

A highly sensitive method for the determination of D-lactate in rat urine was developed by using a high-performance liquid chromatography (HPLC) with an octadecylsilica (ODS) connected to a chiral column. At first, (D+L)-Lactate in the urine were derivatized with a fluorescent reagent, 4-nitro-7-piperazino-2,1,3-benzoxadiazole (NBD-PZ), and separated on the ODS column and determined fluorimetrically at 547 nm with 491 nm of excitation wavelength. During the separation step on the ODS, the peak fraction of (D+L)-lactate derivative was introduced directly to an amylose tris (3,5-dimethylphenylcarbamate) (Chiralpak AD-RH) chiral column by changing the flow of the eluent via 6-port valve. Then, D-lactate derivative was separated enantiomerically from 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 %. The intra- and inter-day precision values were within 0.80 and 14.44 %. The detection limits for D-lactate was approx. 1.0 μM (signal to noise ratio 3).

The proposed method was applied to the urine of diabetic rats induced by intraperitoneal administration of streptozotocin, and the significant increases of D-lactate was observed in the diabetic rats as compared to the normal rats.

Keywords: D-lactate, NBD-PZ, Chiralpak AD-RH fluorimetrically.

二、緣由與目的

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

The present determination methods for D-lactic acid have mostly utilized D-lactate dehydrogenase (D-LDH) [1,2,6-10], which catalyzes the conversion of D-lactate to pyruvate in the presence of NAD^+ to be detected absorptio- [6] or fluorimetrically [2]. Although these methods could determine D-lactate rapidly, the enzymatic reaction is influenced by various endogenous compounds such as fructose 1,6-bisphosphate, 3-phosphoglyceric acid, pyruvate, L-lactate and *S*-lactonyl glutathione [6, 11], which could affect the accuracy and precision of these methods. To overcome the disadvantage mentioned above, an HPLC method using a chiral stationary phase to separate D-lactate from L-lactate following pre-column fluorescence derivatization was

developed. Since very small amount of D-lactate exists with a large excess of L-lactate in the biological samples, we tried to develop a new method to make D-lactate elute first in the chiral separation in order to avoid the peak of D-lactate being covered by that of L-lactate.

In this paper, we investigated the enantiomeric separation of D,L-lactate derivatized with 4-nitro-7-piperazino-2,1,3-benzoxadiazole (NBD-PZ) or 4-(*N,N*-dimethylaminosulfonyl)-7-piperazino-2,1,3- benzoxadiazoles (DBD-PZ) as a pre-column fluorescent reagent (Fig. 1). The D,L-lactate derivatives with NBD-PZ were separated on three 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 Chiralpak AD-RH gives a satisfactory separation and the determination of D-lactate in rat urine by the proposed method was investigated.

三、結果與討論

At first, (D+L)-Lactate in the normal and diabetic rat urine were derivatized with a fluorescent reagent, 4-nitro-7-piperazino-2,1,3-benzoxadiazole (NBD-PZ), and separated on the ODS column and determined fluorimetrically at 547 nm with 491 nm of excitation wavelength (Fig. 3A, 3B). During the separation step on the ODS, the peak fraction of (D+L)-lactate derivative was introduced directly to a polysaccharide-type chiral column by changing the flow of the eluent via 6-port valve. Then, D-lactate derivative was separated enantiomerically from L-lactate derivative, and the enantiomeric ratio was determined from the chromatogram (Fig. 4). For the separation of D-lactate, three polysaccharide-type chiral stationary phases were used. Chiralpak AD-RH gives the best resolution capacity among the three chiral columns: Chiralcel OD-RH, Chiralcel OJ-R, and Chiralpak AD-RH (Tab. 1). The intra- and inter-day precision values were within 0.80% to 14.44%. The accuracy values for the determination of D-lactate in the urine were 96.93-104.85%.

In the three columns, Chiralcel OD-RH and Chiralcel OJ-R are cellulose-chiral stationary phases, whereas Chiralpak AD-RH is an amylose-chiral stationary phase, which gives a satisfactory separation. It may be attributed to the fact that the amylose-chiral stationary phase is more helical in nature and has well-defined grooves making it different from the corresponding cellulose analog, which appeared to be more linear and rigid in nature and hence provides a greater chiral environment for the lactate enantiomers [12,13]. In addition, the enantiomeric separation is dependent on the interactions, including hydrogen bonding and π-π interactions, between the enantiomers and the stationary phases. The kind of interactions was thick as chiral discrimination capability to the enantiomers [12,14]. The carbonyl oxygen on the lactate derivative might form a hydrogen bond with the NH proton on the carbamate moiety. Another hydrogen bonding was formed between the carbamate oxygen and the hydroxyl group located at the chiral center of D-lactate derivative. The resolution of the D,L-lactate derivatived with NBD-PZ was greater on Chiralcel OD-RH and Chiralpak AD-RH than Chiralcel OJ-R chiral column, therefore indicating that OD-RH and AD-RH are better chiral stationary phases than OJ-R in this case. Compare the derivatives on their stationary phases, Chiralcel OD-RH and Chiralpak AD-RH are 3,5-dimethylphenylcarbamate, while Chiralcel OJ-R is 4-methylbenzoate. There is, however, no NH proton on the OJ-R column and the only hydrogen bonding interaction between the carbonyl oxygen and the hydroxyl of D-lactate derivatived with NBD-PZ is weaker. In addition, the presence of the two methyl groups on the phenyl moieties of 3,5-dimethylphenylcarbamate increases the π basicity of the phenyl moieties, which results in π-π interactions of greater magnitude in comparison to Chiralcel OJ-R since there is only one electron-donating methyl group on the benzene ring of the OJ-R. So that 3,5-dimethylphenylcarbamate is more suitable to resolve D-lactate derivatived with NBD-PZ than 4-methylbenzoate [12,14]. But the main interaction force in OJ-R might be the π-π interaction derived from both the original aromatic rings and the 7-piperazino-2,1,3-benzoxadiazole ring introduced by the derivatization. It may be play an important role to resolve D,L-lactate derivatives with NBD-PZ or DBD-PZ on OJ-R, which has larger interaction with D,L-lactate derivatives with DBD-PZ since dimethylaminosulfonyl on DBD-PZ is less electron-withdrawing than nitrosyl group on NBD-PZ, unlike the hydrogen bond are more important on OD-RH and AD-RH. It may be concluded 3,5-dimethylphenylcarbamate is more

suitable to resolve D-lactate derivatived with NBD-PZ and 4-methylbenzoate has a better chiral resolution to D-lactate derivatived with DBD-PZ. When using aqueous methanol as the mobile phase, complete resolution on these columns could not be obtained, and the D-lactate derivatives were retained on the column until the methanol increased to approximate 80%. It might caused by the higher polarity of the mobile phase, which can not elute the enantiomers from the chiral stationary phase, and methanol might intercept the hydrogen binding interactions, resulting in a poor resolution [14].

The inversion of the elution order was also interesting. In our method, the resolved enantiomers were found that the D-lactate enantiomer eluted first, followed by the L-lactate enantiomer in all the cases, whereas in other reversed-phase mode D-lactate enantiomer were all eluted later [15-18]. The inversion of the elution order can solve the problem of large peak of L-lactate cover over the peak of D-lactate when a small amount of D-lactate in a large excess of L-lactate. Considering the researches of D,L-lactate separation [15,17], they used the cyclodextrin- type chiral column to resolve the D,L-lactate derivatives. This kind of chiral stationary phase was similar to amylose, which contains from glucopyranose units bound via α-(1,4)-linkages [19]. The secondary hydroxyl groups at the 2- and 3-positions of the individual glucose units line the "mouth" or wider opening of the cyclodextrin cavity. The primary hydroxyl groups at the glucose 6-position surround the opposite narrower opening of the toroid. Thus the cyclodextrin molecule is shaped like a truncated cone with the secondary hydroxyl side more open than the primary hydroxyl side. While the primary hydroxyl groups on the truncated end can rotate to partially block the cavity, the secondary hydroxyl groups are held relatively rigid. Therefore, the interior is relatively hydrophobic in comparison with polar solvents such as water, while the mouth of the cyclodextrin cavity is hydrophilic in reversed phase mode [12,13,19]. There are several requirements, which are necessary for chiral recognition to occur. Not only must an inclusion complex should be formed between the solute and the cyclodextrin cavity but there should be a relatively "tight fit" between the hydrophobic species and the cyclodextrin cavity. The size of an enantiomer relative to the cyclodextrin cavity is perhaps the most critical parameter governing inclusion complex formation. In other words, if the diameter of the enantiomer is significantly larger or smaller than the mouth of the cyclodextrin cavity, the inclusion complex will not be formed [13,19]. On the other hand, polysaccharides such as cellulose and amylose are the most accessible optically active polymers. These polysaccharides comprise a linear chain of glucopyranose units linked to one another via a 1,4-glycosidic bond and formed a highly ordered helical structure. Their chiral recognition ability may appear due to the higher-order structures of the polymer such as "grooves". Therefore, the enantiomers fit stereogenically in a different fashion into the chiral grooves of the stationary phases, hence the resolution of enantiomers occurred [12,14,20]. Compare the different mechanisms of cyclodextrins and polysaccharides, cyclodextrins formed inclusion complex with solute in its cavity and polysaccharides interact with solute on the chiral helical groove. Chiral recognition by the cyclodextrin are quite different from the polysaccharides, the higher-order structure of the cyclic oligosaccharides must be different from that of a linear polysaccharides [20]. However, the mechanism of the inverted interaction of D,L-lactate with cellulose and amylose against cyclodextrin is still unclear. The hypothetic reason for the inverted elution of enantiomers is considered to be as follows. In cyclodextrin, D-lactate is more fit the cavity and form stronger interaction with cyclodextrin than L-lactate. On the other hand, this result suggests that L-lactate might strong interact with polysaccharide, i.e. L-lactate might go smoothly into the grooves and be better accommodated than D-lactate [21]. The conformation of L-lactate maybe recognize by two grooves on polysaccharide stationary phase, which form more interaction between solute and column. In other words, D-lactate interacts with polysaccharide stationary phase in the first groove similar with cyclodextrin, and form other bonding with the second groove on polysaccharide. Because of the different steric effect of D- and L-lactate on stationary phase, the first groove provides greater hydrogen bond with D-lactate and the second groove provides another greater hydrogen bond with L-lactate enantiomer than D-lactate. Considering the reason mentioned above, the inversion of the elution order of lactate enantiomers was occurred. But further experiments and discussion are necessary for fully interpreting the resolution mechanism.

The proposed HPLC method was applied to determine D-lactate in the urine of normal and

diabetic rats. Figure 5 shows the mean ± SD of D-lactate/creatinine ratio in the urine of normal and diabetic rats. The ratios of D-lactate/creatinine significantly increased as compared to those of normal rats. The studies of the relations of D-lactate concentration to diabetic complications such as renal dysfunction remains to be carried out, we have developed the method for the highly sensitive determination of D-lactate in 20 μL of rat urine .

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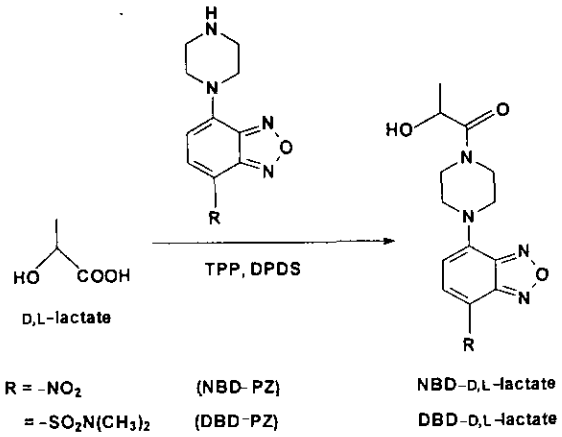

Figure 1. Derivatization scheme of lactic acid with NBD-PZ or DBD-PZ

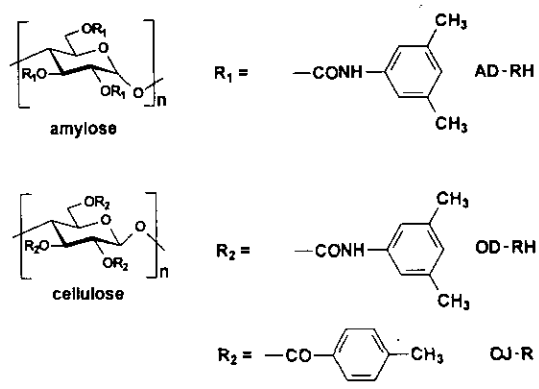

Figure 2. Structures of the chiral stationary phases Chiralcel OD-RH, Chiralcel OJ-R and Chiralpak AD-RH.

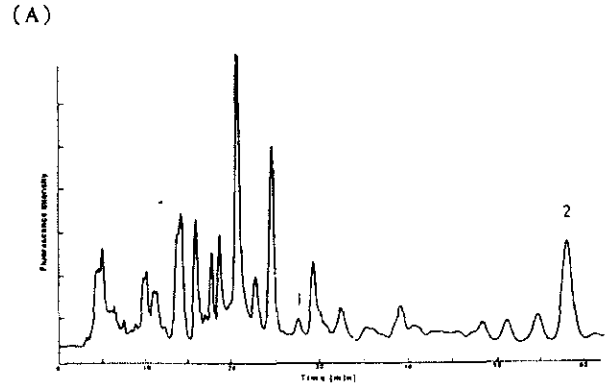

Figure 3. Chromatograms of samples from the urine of normal (A) and diabetic rats (B) derivatized with NBD-PZ separated on the ODS column. 1; lactate derivative, 2; propionate derivative (I.S.).

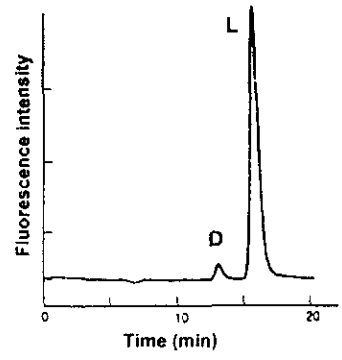

Figure 4. The chromatograms of resolved enantiomers of lactate derivatized with NBD-PZ on Chiralpak AD-RH column using mobile phase $CH_3CN : H_2O = 60 : 40$

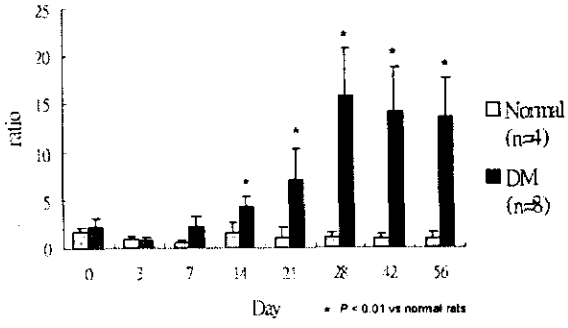

Figure 5. Ratio of D-lactate and creatinine in the urine of normal and diabetic rats. *p<0.01 vs. normal rats.

Table. 1 Effect of mobile phase compositions on capacity factors ($k1$, $k2$), separation factor (α) and resolution (Rs) of the D,L-lactate derivative with NBD-PZ

mobile phase composition		Chiralcel OD-RH				Chiralcel OJ-R				Chiralpak AD-RH			
		k_1	k_2	α	Rs	k_1	k_2	α	Rs	k_1	k_2	α	Rs
MeCN:H_2O	20:80	15.4	16.2	1.05	1.06	4.06	4.06	1	0	n.d.	n.d.	n.d.	n.d.
	40:60	1.64	1.64	1	0	0.46	0.46	1	0	3.29	4.61	1.4	3.07
	60:40	0.59	0.59	1	0	0.11	0.11	1	0	1.19	1.7	1.43	2.63
	80:20	0.3	0.3	1	0	0.1	0.1	1	0	0.8	1.14	1.43	1.83
MeOH:H_2O	40:60	n.d.	n.d.	n.d.	n.d.	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.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	80:20	5.33	5.59	1.05	0.43	2.26	2.26	1	0	n.d.	n.d.	n.d.	n.d.
MeCN:MeOH:H_2O	30:20:50	2.51	2.51	1	0	0.67	0.67	1	0	n.d.	n.d.	n.d.	n.d.
	75:20:05	0.31	0.31	1	0	0.19	0.19	1	0	n.d.	n.d.	n.d.	n.d.