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The Development of a Column-Switching High-Performance Liquid Chromatograph System for Chiral Separation of Mouse Urinary D, L-Lactate

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Abstract

Two high-performance liquid chromatograph (HPLC) systems with a Rheodyne Model 7000 switching valve for separation of D- and L-lactate in biological samples has been reported. However, since the switching valve was rotated manually, so that only 10 samples could be determined within one day. For better efficiency, a circuit for rotating the switching valve automatically, which is including mechanical units of motion, two timers, and electronic control units was designed. Total (D+L)-lactate fluorescent derivatives was separated from others by a TSKgel ODS-80Ts column, when the peak of lactate derivatives appeared in the chromatogram on the ODS column, the valve position was changed by switching and then the isolated lactate derivatives was introduced into the chiral column automatically. Then the D- and L- enantiomers were separated by the chiral column. The utility of the analyzer system was tested for the determination of chiral separation of urinary D, L-lactate in the mouse. It takes about 90 min to analyze one urine sample for a circulation. Before automation, we must turn the switching valve manually when the signal shows up, so that there must be someone always standby and only about 10 samples could be analyzed within one day. With the designed automatic column-switching HPLC system, the switching valve can be automatically turned at the setting time repeatedly and over 15 samples could be determined within one day. The automatic system makes the analysis of D-lactate be time-saving, convenient and reproducible.

Keywords: *Column-switching HPLC; Switching valve; Automation; Chiral separation; D-lactate.*

1. Introduction

High-performance liquid chromatography (HPLC) is a popular analytical method and is commonly used for the separation of chemical compounds. For investigating the role of D-lactate in mammals, we developed highly sensitive HPLC methods for determination of D-lactate in biological samples like rat serum [1-3], urine [4], and human serum [5]. The proposed methods were further applied to the plasma [2] or urine [4] of diabetic rats induced by intraperitoneal administration of streptozotocin, and the significant increases of D-lactate concentrations was observed in the diabetic rats as compared to the normal ones. In diabetic rats, D-lactate concentrations revealed a rising trend from the 7th day and then kept stable from the 28th day after induction, suggesting that urinary D-lactate may be used as a marker to determine the diabetic stage and the level of kidney damage [4]. Significantly increased D-

lactate and L-lactate concentrations were also observed in the serum of diabetic patients as compared with normal subjects [5]. As mentioned above, the HPLC methods are effective in analyzing D-lactate concentration under physiological or pathological conditions.

All the HPLC systems used in the above methods contained two HPLCs with a switching valve, as illustrated in Fig. 1. Total (D+L)-lactate fluorescent derivatives was isolated by an ODS column in first HPLC and then the isolated lactate derivatives was introduced into the chiral column by rotating the switching valve manually. Then the D- and L-enantiomers were separated on the chiral column. It takes 34 min to obtain the total (D+L)-lactate, and an extra 20 min for washing column and another 30 min for returning to the initiate sate. It takes average time of one and a half hours to complete a separation circle. If the designed automatic valve were not set in the separation system, the switching valve must be turned manually when the signal shows up, so that there must be someone always standby and only about 10 samples could be analyzed within one day at most. Although there are many two-dimensional HPLC systems in the market, all of them are the type of time setting. In the study, we desired to construct a simple, cheap automatic switching valve that can be used in our existing HPLC system.

In the present study, we designed an automation system to rotate the switching valve when total (D+L)-lactate is just isolated, and drive the second integrator to record the chromatogram. In Fig. 1 the relevant coordinate system and parameters of a valve are shown. With the designed automatic column-switching HPLC system, the investigation of the correlation between D-lactate concentration in mouse urine and the stage of kidney damage will speed up.

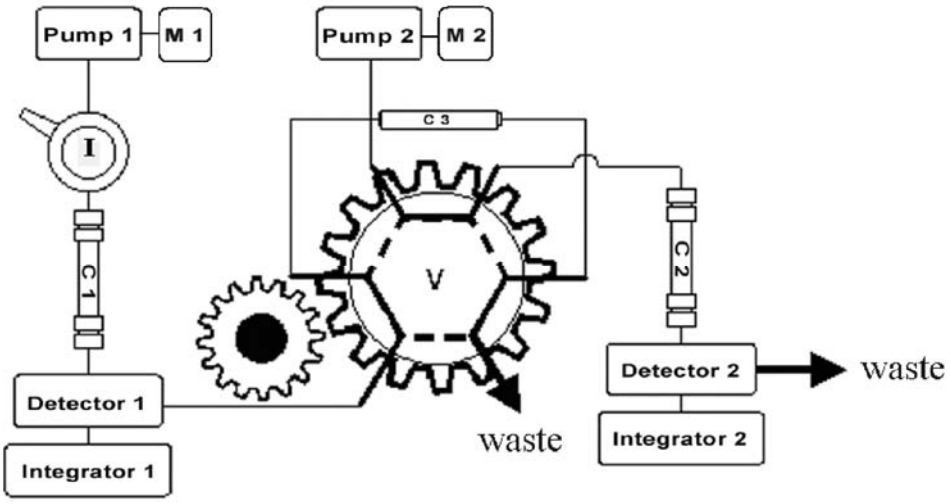

Figure 1. A schematic diagram for the automatic column-switching HPLC system used in this study was shown. The solid and dotted lines indicate switching valve position A and B, respectively. Abbreviations used are as follows: C1, column 1 (TSKgel ODS -80Ts); C2, column 2 (Chiralpak AD-RH); C3, column 3 (COSMOSIL® 5C18-MS-Ⅱguard column); M1, H_2O/MeOH/CH_3CN (70/20/10); M2, H_2O/CH_3CN (40/60).

2. Experimental

Animals

Animal experiments were approved by Laboratory Animal Research Committee of Taipei Medical University. Female C57BL/6 mice (Laboratory Animal Center of National Taiwan University, Taipei, Taiwan) were used in the experiment and kept in an environmentally controlled room with food and tap water ad libitum. To induce Nephrotoxic Serum (NTS) nephritis, C57BL/6 mice were immunized with 250 μg of rabbit IgG and 0.05 mL of Freund Complete Adjuvant (FCA) in the rear footpad. Five days later, mice were intravenously injected with 100 μL of NTS. The urine was collected using metabolic cage once a week for 12 hrs.

Samples and Derivatization procedure

Urinary D- and L-lactate were derivatized with 4-nitro-7-piperazino-2,1,3-benzoxadiazole (NBD-PZ) according to the previous study[1] as follows: 100 μL of mouse urine was added to 400 μL of CH_3CN then centrifuged, then 100 μL of the supernatant was added to 50 μL of 10 mM NBD-PZ in CH_3CN in the presence of 50 μL each of 280 mM triphenylphosphine and 2,2'-dipyridyl disulfide in CH_3CN. After standing for 3 h at 30℃, 250 μL of 0.1% trifluoroacetic acid in H_2O was added to stop the reaction. In order to remove the excess fluorescent reagent 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 and filtered with a 0.22 μm syringe filter. Twenty microliters of the elute solution was injected into the HPLC.

HPLC conditions

A column-switching HPLC system was used in the present work. As illustrated in Figure 1, the HPLC system was equipped with a AS-950 intelligent sampler (Jasco, Tokyo, Japan), two pumps (L-7100; Hitachi, Tokyo, Japan), an F-1000 and an L-7485 fluorescence detector (Hitachi), two D-2500 Chromato-Integrators (Hitachi), and a Rheodyne Model 7000 switching valve (Rheodyne, Rohnert Park, CA., USA) with a COSMOSIL® $5C_{18}$-MS-Ⅱ guard column (Nacalai Tesque, Kyoto, Japan) as a sample trap. A TSKgel ODS-80Ts column was used for isolation and quantification of the total (D+L)-lactate in mouse urine; the mobile phase was H_2O/MeOH/CH_3CN (70/20/10, v/v) at a flow rate of 0.7 mL/min. A column packed with amylose tris (3,5-dimethylphenylcarbamate) coated on silica gel (Chiralpak AD-RH; Daicel, Osaka, Japan) was used for the chiral separation of D- and L-lactate eluted with H_2O/CH_3CN (40/60, v/v), and the flow rate was 0.3 mL/min. Fluorescence detection (detectors 1 and 2) was performed at 547 nm with a 491 nm excitation wavelength [1].

The integrator received the fluorescence intensity and transform it into voltage (0~1000 μv) to recorder. According to the information given from integrator, the recorder printed out the chromatograms. As the monitored peak of total lactate appeared on the chromatograms in Integrator 1, eluted total (D+L)-lactate was presumed to be isolated in the sample trap column, and the valve position must be switched from A (solid line) to B (dotted line) to introduce the total (D+L)-lactate into the chiral column. Then, the D and L isomers were separated

enantiomerically on the chiral column, and enantioseparation chromatograms were obtained from Integrator 2.

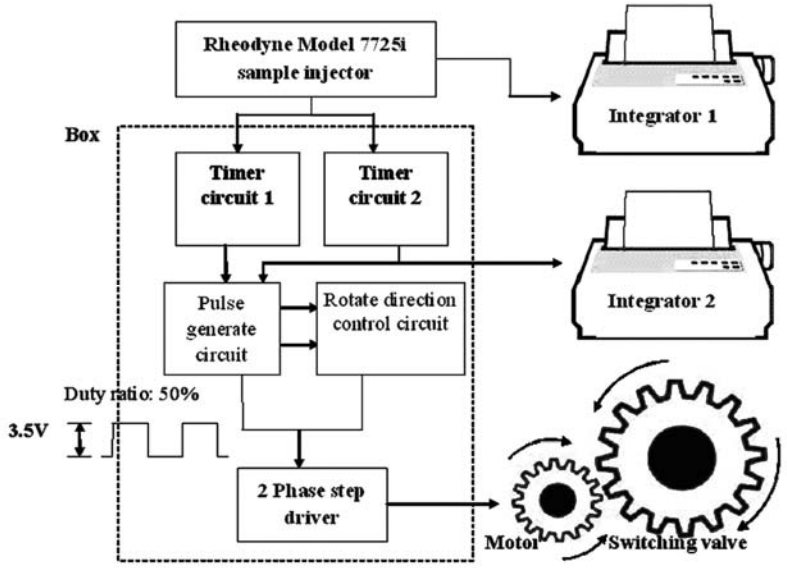

Figure 2. The Electric hardware blocks diagram for denoting the designed relevant system.

Mechanical Design in switching valve

A two phase step motor (TS3617N3E8; TAMAGAWA, Japan) was used to rotate the switching valve in this study. As illustrated in Figure 2, they were connected by a coupling with gear. The holding torque of step motor was 3.2 kg·cm and was operated in half step (0.9°/step), 0.2 KPPS.

Circuits

The switching valve must be switched from A (solid line) to B (dotted line) of Figure 1 at the setting timing. Circuits can be divided into three parts: timer circuit and pulse generate circuit and rotate direction control circuit.

a. Timer circuit

Two commercial countdown circuit suites were used in this study (VCT-TDS; Tun-Hwa Electronic Material, Taiwan). Each suite mainly consists of an already programmed 89C51 to do the countdown operation; six seven-segment displays showing the time and one relay which can be used for advanced control. The relay was employed to generate a ground signal with 1 second period for triggering the pulse generated circuit. Before analyzing biological samples, we determined the retention time of racemic D, L-lactate by injection of standard. Because different chemical was isolated in different time, we designed a timer circuit to determine which compound we want to deal with.

In this case, we want to separate the total (D+L)-lactate into D and L enantiomers. According to the chromatogram of standard, we know the total (D+L)-lactate appeared around 34 min. When sample injector injected biological samples into the TSKgel ODS-80Ts column,

it also produced a short signal and we took it to be the start signal of our system. Once the timer circuits received the start signal, they began to count down. Timer 1 was set at the time when total (D+L)-lactate was going to be isolated and timer 2 was set at 4 minutes after time 1. Four minutes was the approximate time from the emergence to disappearance of the (D+L)-lactate, it might be gotten by preliminary experiments although it is different by chemicals. When timer circuit 1 counted to zero, switching valve was turned from position B to position A to introduce total (D+L)-lactate into the guard column. Four minutes later, total (D+L)-lactate was isolated completely. Timer circuit 2 would count to zero to turn the valve back from A to B and start the integrator 2 at the same time. Then the D and L enantiomers were separated enantiomerically on the chiral column. When two timer circuits counted to zero, they started pulse generate circuit to produce square waves to drive stepping motor.

b. Pulse generate circuit and Rotate direction control circuit

Pulse generate circuit was constructed mainly by a timer IC (STMicroelectronics, NE555N) and three synchronous up/down decade counters (STMicroelectronics, M74HC192). Timer IC aimed at producing square waves with 3.5V amplitude to drive the motor while counters and dip switches defined the number of waves which were required. The angle difference of switching valve between position A and B was 58°. Due to stepping motor must switch the valve from position A to position B, the pulse generate circuit produced square waves with 50% duty ratio at 200 Hz each time to drive the motor to rotate switching valve after timer circuits count down. After each rotation, pulse generate circuit sent a signal to the rotate direction control circuit to make stepping motor always rotates in reverse direction at next time. In this way, we can also prevent the switching valve from being damaged if there was a mis-trigger driving the pulse generate circuit.

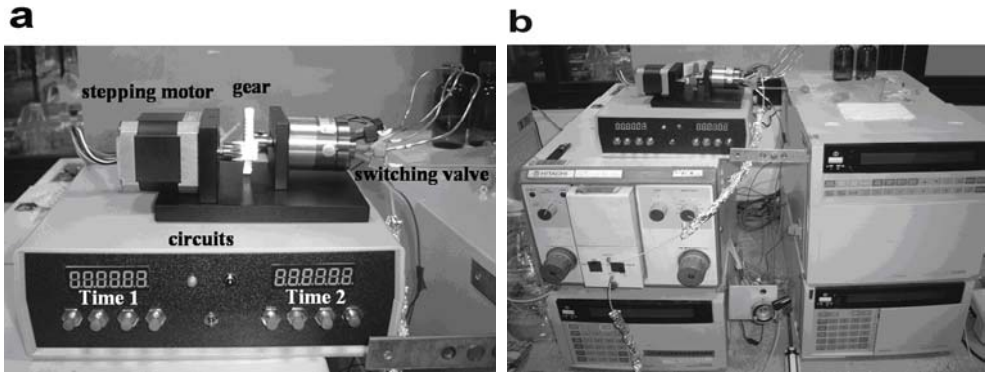

Figure 3. (a) The hardware of the connection for gear coupling, step motor and switching valve. (b) All views of automated column-switching HPLC system.

3. Application

The hardware of the automated switching valve and the HPLC system are shown in the figure 3a and figure 3b, respectively. The proposed column-switching HPLC method was applied to the urine of normal and renal failure mice. Urinary D, L-lactate reacted with NBD-PZ for fluorescent derivatization, and was separated on the ODS column and determined fluorimetrically at 547 nm with 491 nm of excitation wavelength (Fig. 4a, b and c). During

the separation step on the ODS column, the peak fraction of (D+L)-lactate derivatives was introduced into an amylose-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. Using this proposed HPLC method, 20 μL of urine sample was sufficient for D-lactate determination. As shown in Fig. 4b and 4c, urinary D-lactate showed a rising trend. Urinary D-lactate may be used as a marker to screen the stage of kidney damage.

Previously, we analyzed the samples with the valve rotated manually, and only about 10 mouse urine samples could be determined within one day. After testing our automatic column-switching HPLC system, we can successfully rotate the valve at the right time, not only in the analysis of normal mice urine, but also in the renal failure mice.

In the future, we will add the voltage slope detect circuits for making the determination of biological samples automatically. When the slope of chromatogram intensity is plus for a time ΔT, the step motor rotate. Second, we need some protect circuit to prevent the switching valve from being over-rotated and damaged.

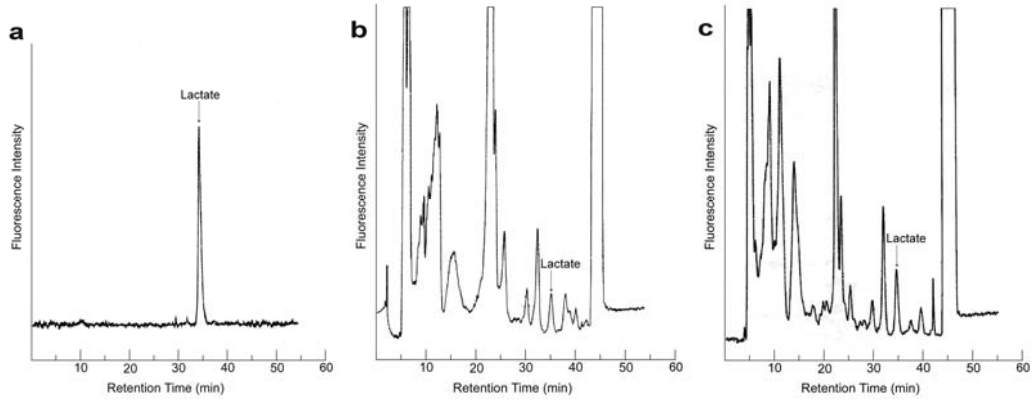

Figure 4. HPLC chromatograms of (D+L)-lactate in normal and renal failure mouse urine, which obtained by proposed automatic column-switching HPLC system. Total (D+L)-lactate was first isolated from standard (a), normal (b) and renal failure mouse urine (c), respectively.

4. Conclusion

With the automatic column-switching HPLC system, more samples could be analyzed within one day and researchers don't need to stay around the machine. It is a useful system especially when great amount of samples have to be analyzed. This automatic system makes the determination of biological samples become more convenient and may be helpful to the researchers.

Acknowledgements

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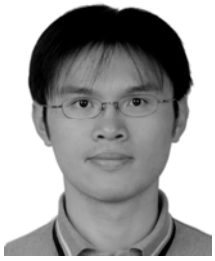

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