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Short communication

Simultaneous determination of L- and D-lactic acid in plasma by capillary electrophoresis

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Abstract

A novel method for simultaneous determination of D- and L-lactic acids in plasma was presented by capillary electrophoresis with photodiode array detection at 195 nm. The separation was performed in an uncoated fused-silica capillary. The parameters influencing the resolution and the migration time of lactic acids were optimized. When 150 mM phosphate–Tris buffer (pH 7.0) consisting of 220 mM 2-hydroxypropyl-β-cyclodextrin and 0.2 mM tetradecyltrimethylammonium bromide was utilized as the running buffer, highly effective chiral separation of D- and L-lactic acids was achieved at about 42 min at an effective voltage of −25 kV. The resolution of lactic acid enantiomers was ≥1.25. The limits of detection of D- and L-lactic acids in standard solution without any pretreatment were 80 and 50 μM (S/N = 3), respectively. Sample pretreatment was preceded by protein-removal procedure with acetonitrile. With a pre-concentration procedure by 10 times, the limits of detection of D- and L-lactic acids were 20 and 15 μM (S/N = 10), respectively. The satisfactory analytical performance of the proposed method was validated.

Keywords: D-Lactic acid; L-Lactic acid; Capillary electrophoresis; Plasma; Simultaneous determination

1. Introduction

Lactic acid is mainly composed of the L-form in mammals, which is produced from pyruvic acid under anaerobic conditions. In addition, L-lactic acid may also be produced by bacterial fermentation in the gastrointestinal tract. In mammals, L-lactic acidosis is most commonly caused by increased peripheral production in tissues due to hypoxia from poor tissue perfusion or by decreased hepatic metabolism of lactic acid [1–4]. But the optical isomer of L-lactic acid, D-lactic acid, also exists in mammals even though its amount is approximately 1.0% relative to L-lactic acid. D-Lactic acid, found in human physiologic fluids, generally is produced from the metabolism of carbohydrate by the action of D-lactate dehydrogenase in presence of intestinal bacteria [5,6], is ingested in foods and absorbed from bacteria in the gut [7], and formed by the glyoxalase pathway [8]. Bacterial overproduction of D-lactate in the gut has mostly been described in patients with short bowel syndrome or after jejunoileal bypass surgery, which leads to D-lactic acidosis associated with encephalopathy [9,10]. It has also been reported that D-lactic acid concentration significantly increases in the serum of diabetic animals [11,12] and patients [13]. Thus, D-lactic acid has the potential to be a clinical marker for the diagnosis of these diseases. Recently, D-lactate level in plasma has been used as a marker of increased intestinal permeability after severe injuries [14]. Both isomers of lactic acid can contribute to metabolic acidosis, but their relative contributions are distinguishing due to their different origins and metabolic pathways. Therefore, it is important to distinguish the isomers of lactic acid in plasma for understanding

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their relative contribution to metabolic acidosis. However, because of the similar physical and chemical properties of both enantiomers, it is difficult to determine the isomers of lactic acid in biologic samples [15]. Thus, it is necessary to develop a sensitive and reliable method for the separation and quantification of lactic acid enantiomers in biologic samples.

The determination of lactic acid is usually based on the enzymatic reaction between lactic acid and co-factor NAD^+. Although the enzymatic assay provides a rapid determination and has been widely used in many laboratories, some disadvantages, such as cross-reaction with endogenous compounds [16] and large sample consumption, have still remained. Rapid and simple chromatographic techniques with chiral ligand-exchange phases or chiral fluorescent derivatization [17–20] can overcome some of the shortcomings of enzymatic analyses. But, most of these methods are seldom applied in biological samples. Moreover, they require different derivatization conditions depending on the organic acid analyzed, because some of them undergo racemization, cyclication or other effects during the sample treatment [21–23]. Recently, a determination method for serum D- and L-lactic acids has been developed by column-switching high-performance liquid chromatography (HPLC) with pre-column fluorescence derivatization [24]. This method can be applied to determine simultaneously two lactic acid enantiomers in normal subjects and diabetic patients. However, the amylose-based chiral column used in the reported method is expensive, and the procedure contains a column switch step and the fluorescence derivatization of lactic acids. Obviously, it is necessary to find one simple, fast and more convenient method for simultaneous determination of serum D- and L-lactic acids.

Capillary electrophoresis (CE) has shown to be a good choice for enantiomeric resolutions using chiral selectors in the separation buffer, and thus can provide a very simple and automated method [25]. The small sample volume needed for injection and the reduced sample pretreatment are the advantages of CE for body fluids analysis. Cyclodextrins and their derivatives or modified crown-ethers have been successfully applied in CE for the enantiomeric separation of a wide number of analytes [26–28]. The separations of aliphatic α-hydroxy acids, including D- and L-lactic acids, have also been achieved by using 2-hydroxypropyl-β-cyclodextrin (2HP-β-CD) [29]. The direct CE chiral separations of lactic acids in food products [30] and body fluids [31] have been reported by using 2HP-β-CD as chiral selector to change the resolution of lactic acid enantiomers and polyvinylalcohol (PVA)-coated bubble cell capillary [30] and polyacrylamide-coated capillary [31] to suppress the electroosmotic flow and avoid the longer migration time with a fused-silica capillary. The problems in these two methods are the coated capillaries cannot be washed with acidic and alkaline solution. The main objectives of this paper are to develop a new CE system to solve the problems described earlier. By adding of tetradecyltrimethylammonium bromide (TTAB) that acts as dynamic coating in the running buffer containing 2HP-β-CD to reverse the electroosmotic flow, the direct CE chiral separation of lactic acids in plasma is obtained with an untreated capillary, which has successfully been used to detect L- and D-lactic acids in plasma. This method could be applied to investigation of acidosis resulted from D-lactic acid in animals and patients with intestinal diseases such as short bowel syndrome and blind loop syndrome and to monitoring increased intestinal permeability after severe injuries.

2. Experimental

2.1. Reagents

Tetradecyltrimethylammonium bromide, 2-hydroxypropyl-β-cyclodextrin (average degree of substitution, 7), D- and L-lactic acids were purchased from Sigma (St. Louis, MO, USA). Phosphoric acid 85% (AR) was obtained from Nanjing Chemistry Reagent Factory (Nanjing, China). Tris-(hydroxymethyl) aminomethane (Tris, GR) was purchased from Shanghai Chemical Reagent Company (Shanghai, China). Acetonitrile (AR) was from Merck (Darmstadt, Germany). Deionized water prepared with Milli-Q-purified water (Millipore, Milford, MA, USA) was used for all experiments.

2.2. Buffer preparation

The running buffer in the electrophoretic experiments, unless stated otherwise, was 150 mM phosphate–Tris buffer (pH 7.0) containing 0.2 mM TTAB and 220 mM 2HP-β-CD, which was adjusted to pH 7.0 with 0.33 M Tris and filtered with a 0.22-μm filter before use. Stock solutions of 30 mM D- and L-lactic acids were individually prepared in deionized water, stored at −20 °C and diluted to 1.0 mM before use.

2.3. Capillary electrophoresis system

Electrophoretic experiments were carried out using a capillary electrophoresis P/ACE^{TM} MDQ (Beckman, Palo Alto, CA, USA) with photodiode array detection at 195 nm. The injection was carried out by a pressure of 0.035 bar for 100 s. The separation was performed with an uncoated fused-silica capillary (50 μm i.d., Ruifeng Company, Hebei, China). The total and effective length was 63 and 55 cm, respectively. The operated voltage was −25 kV. Temperature was maintained at 18 °C. In order to reverse electroosmotic flow (EOF), 0.2 mM TTAB was added into the running buffer. Between two consecutive experiments, the capillary was rinsed sequentially with 1.0 M sodium hydroxide (2 min), water (3 min) and then the running buffer (3 min). These conditioning steps improved the repeatability of the electroosmotic flow.

2.4. Sample preparation

Venous blood samples, anticoagulated with 0.3% heparin sodium, were obtained from human and rat. The plasma was prepared by centrifuging the sample at 1600 × *g* for 20 min and stored at −70 °C. Plasma samples were prepared by mixing 750 μl acetonitrile with 250 μl plasma samples on a vortex for 200 s and centrifuging the mixture at 8000 × *g* for 30 min. The 900 μl supernatant solutions were then evaporated with SPD1010 Speed-Vac® System (Thermo Savant, USA). The residues were redissolved in 25 μl deionized water for CE determinations.

2.5. Enzymatic assay for D-lactic acid

Enzymatic assay was performed according to the literature method [16] with minor modification. Briefly, 0.1 ml of ice-cold perchloric acid (5.8 M) was added to 1.0 ml of human plasma. After mixing the resulting solution on a vortex mixer for 1 min, the solution was kept on ice for 10 min. The solution was then centrifuged at 2000 × *g* for 10 min at 4 °C, 0.9 ml of supernatant was transferred into another tube, and neutralized with 0.06 ml of potassium hydroxide (5.8 M), and then the pH was adjusted at 10–12 with this solution. After 10 min in an ice bath, the solution was centrifuged for 10 min at 2000 × *g* and 4 °C. A 0.6 ml obtained supernatant was added to 1.8 ml NAD^+ (1.67 mg/ml)–glycine (12.52 mg/ml)–hydrazine solution (0.58%, v/v), and 1 ml the mixed solution was added to each of two tubes. The resulting solution was incubated for 120 min at 25 °C after adding 0.05 ml D-lactic dehydrogenase (D-LDH) (600 units/ml). D-Lactic acid concentrations were estimated as UV absorption spectra of NADH measured at 340 nm using a DV800 spectrophotometer (Beckman Coulter, USA).

3. Results and discussion

3.1. Optimization of electrolyte parameters

3.1.1. Effect of buffer type and buffer concentration

The physicochemical properties such as buffering range, running current, and background absorbance need to be considered when choosing a CE running buffer. This work examined 10 and 20 mM sodium borate buffers (pH 9.2), 50, 100, 150 and 200 mM sodium phosphate buffers (pH 6.0), respectively, and phosphate–Tris buffer at pH 6.0. Lactic acid enantiomers were not separated in those sodium borate buffers, which probably was due to the formation of a borate–cyclodextrin complex [32]. Sodium phosphate buffers exhibited a high running current, which consequently resulted in severe peak dispersion due to Joule heating. The high concentration of Tris did not produce a large running current due to its large ion mass and weak electrolyte property. This allowed a higher concentration of phosphate–Tris buffer to be used. Fig. 1A shows the effects of concentration of phosphate–Tris buffer on the resolution (R_s) and the migration time of lactic acid enantiomers. With an increasing

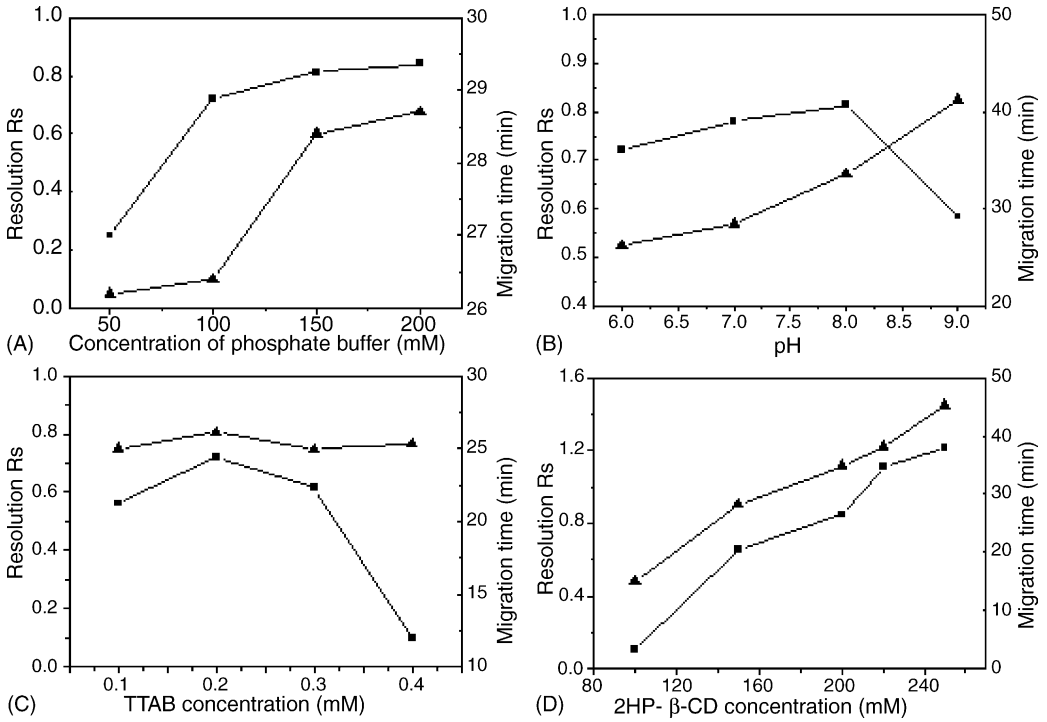

Fig. 1. Effects of phosphate–Tris buffer concentration (A), pH (B), TTAB concentration (C) and 2HP-β-CD concentration (D) on D- and L-lactic acid resolution and migration time. When one parameter changed other conditions were 150 mM phosphate–Tris buffer (pH 6.0) containing 150 mM 2HP-β-CD and 0.2 mM TTAB at −20 kV and 20 °C. Concentration of D- and L- lactic acids was 1.0 mM, respectively. (■) Resolution; (▲) migration time.

concentration of phosphate–Tris buffer, both resolution and migration time increased and then leveled off a maximum at 150 mM. Thus, 150 mM phosphate–Tris buffer was therefore chosen as the running buffer.

3.1.2. Effect of buffer pH

Fig. 1B shows the effects of pH of the running buffer on the resolution and migration time of lactic acid enantiomers. The pH was adjusted by adding Tris in phosphate buffer. No peak of lactic acid was observed in experimental time range at pH $\leq$5 due to the lactic acid dissociation constant (pK_a) of 3.86, which led to the coexistence of lactic acid ion and lactic acid molecule in electrolyte and thereby slower mobility. When the pH was adjusted from 6 to 8, R_s value increased slightly and migration time was also prolonged. Upon further increase of pH, R_s value decreased sharply, while migration time further increased. Considering both resolution and migration time the final separation of analytes was performed at pH 7.0.

3.1.3. Effect of TTAB concentration

Since the carboxyl group of lactic acid (pK_a = 3.86) was negatively charged at pH 6–9, the analyte migrated electrophoretically to the anode. In general, the apparent migration depends on the net difference between the two driving forces, electrophoretic mobility (EM) and electroosmotic mobility (EOM). When the EM of the anionic analyte is close to or smaller than the EOM, the anionic analyte is difficult or even impossible to be detected due to the counteraction between electroosmotic flow and EM. However, reversed EOF can shorten migration time, which can be easily realized by adding cationic surfactants in running buffer [33], due to the adsorption of cationic surfactants on the charged surface of the silica capillary to form a primary hydrophobic layer by electrostatic interaction and then a bilayer by hydrophobic interaction [34,35].

Tetradecyltrimethylammonium bromide is a kind of cationic surfactant. When TTAB (>0.05 mM) was added in the buffer, the electroosmotic flow reversed towards the positive pole, avoiding the longer migration time with a fused-silica capillary. Fig. 1C shows the effects of TTAB concentration ranging from 0.1 to 0.4 mM on the resolution and migration time of lactic acid. The concentration of TTAB showed obvious influence on the resolution. When the concentration of TTAB was changed from 0.1 to 0.2 mM, the resolution increased gradually, however, once the concentration was higher than 0.2 mM, the resolution decreased dramatically due to peak tailing. So, 0.2 mM TTAB concentration was considered as the optimum concentration. In general, with the increasing concentration of TTAB in running buffer the EOM changes in both its direction and its value. After reversal, the EOM increases with increasing TTAB concentration, thus the migration time is shortened. However, in our experiment, the concentration of TTAB showed a little effect on the migration time. It seemed that more than one factors affected the migration time, including ion-pairing and/or adsorptive interaction of the acidic solute with TTAB in electrolyte and wall-coating TTAB, respectively. In additional, as shown in Fig. 1D, 2HP-β-CD in electrolyte also showed an obvious effect on the migration time. The presence of 2HP-β-CD played an important role in controlling the migration time.

3.1.4. Effect of cyclodextrin concentration

As known, 2HP-β-CD is effective for the chiral resolution of aliphatic α-hydroxy acids containing lactic acid using CE [29,30]. When the 2HP-β-CD concentration was lower than 50 mM, no resolution of D- and L-lactic acids was observed, and when it was higher than 250 mM, the migration time of analyte was so long that peak was severely dispersed. The effects of 2HP-β-CD concentration ranging from 100 to 250 mM on the resolution and the migration time of lactic acid are shown in Fig. 1D. With an increasing amount of 2HP-β-CD, the resolution and the migration time enhanced. Considering the migration time and the peak shape, the 2HP-β-CD concentration of 220 mM was used for sample detection. Upon complexation with the cyclodextrin, the electrophoretic mobility of lactic acid was reduced, because of the larger hydrodynamic radius of the complexed solute as compared to the free solute that possessed similar effective charge. By increasing the concentration of the cyclodextrin, a higher molar fraction of the solute was present in the complexed form so that migration times increased with increasing cyclodextrin concentrations. The need of a high concentration of 2HP-β-CD in the proposed CE system indicated a weak interaction between 2HP-β-CD and lactic acid. The D-lactic acid moved more slowly than L-lactic acid, so D-lactic acid formed a stronger diastereomer complex with 2HP-β-CD than L-lactic acid. 2HP-β-CD possessed no self-electrophoretic mobility and moved with EOF. This dependency between migration time and CD concentration was also reported in the cases of other enantiomer–CD complexes [31].

In summary, the optimized CE separation was performed with 150 mM phosphate–Tris buffer (pH 7.0) containing 220 mM 2HP-β-CD and 0.2 mM TTAB with an effective voltage of −25 kV at 18 °C. These conditions were applied to the practical rat and human plasma samples. The resolution and running time at these conditions were comparable with those reported previously [31].

3.2. Linearity, detection limit of the lactic acid enantiomers

D- and L-lactic acid stock solutions (30 mM) were diluted to the concentration range of 0.025–2.50 mM for D-lactic acid and 0.025–5.0 mM for L-lactic acid. After treated by mixing 250 μl standard solutions with 750 μl acetonitrile and centrifuging the mixture at 8000 × g for 30 min followed with a concentration step, the samples were injected to the CE system for concentration determinations under the optimum conditions. The calibration curves for lactic acid showed the excellent linearity. The regression equations were $y = 40036x - 461.8$ and $y = 54232x - 915.8$ with

Table 1
Precision (R.S.D., %) and accuracy (%) for D- and L-lactic acid in plasma

Analyte	Spike level (mM)	Intra-day R.S.D. (%, $n = 3$)		Inter-day R.S.D. (%, $n = 3$)		Recovery (%)
		Peak area	Migration time	Peak area	Migration time	
Human plasma						
D-Lactic acid	0.05	5.22	3.41	6.10	2.10	68.2 ± 4.2
	0.2	1.83	2.47	3.14	2.66	75.0 ± 3.8
	1.0	6.27	4.26	4.67	0.73	74.1 ± 2.2
L-Lactic acid	0.05	5.07	3.11	6.00	2.11	71.3 ± 4.3
	0.2	5.38	2.55	3.78	2.77	70.3 ± 6.5
	1.0	6.02	4.23	5.32	0.71	75.1 ± 2.8
Rat plasma						
D-Lactic acid	0.05	5.03	1.67	4.98	1.01	75.2 ± 6.7
	0.2	7.28	1.57	4.72	0.59	71.5 ± 5.2
	1.0	7.13	1.28	6.50	1.10	88.6 ± 0.01
L-Lactic acid	0.05	2.13	1.66	3.72	1.04	86.2 ± 3.4
	0.2	1.27	1.54	0.54	0.59	79.7 ± 2.0
	1.0	0.91	1.24	0.80	1.00	82.9 ± 0.03

the correlation coefficients of 0.9969 and 0.9952 for D- and L-lactic acids, respectively.

Both peaks of D- and L-lactic acids were clearly observed in the electropherograms at the D- and L-lactic acid concentrations of approximately 80 and 50 μM (S/N = 3), respectively. With a concentration step described in Section 2.4, the limits of detection of D- and L-lactic acids were 20 and 15 μM (S/N = 10), respectively.

3.3. Validation of proposed method

When the human and rat plasma samples contained 0.05, 0.2 and 1.0 mM D- and L-lactic acids, the recoveries for determinations of D- and L-lactic acids with the sample preparation procedure described in Section 2.4 were from 68.2% to 75.1% in human plasma and from 71.5% to 88.6% in rat plasma (Table 1).

The precision of the proposed method was examined at 0.05, 0.2 and 1.0 mM D- and L-lactic acid concentrations. The results are listed in Table 1. The relative standard derivation (R.S.D.) was from 1.83% to 7.28% and 0.91% to 6.02% for intra-day assays of peak areas of D- and L-lactic acids, respectively. The intra-day assays of migration time showed the R.S.D. of 1.28–4.26% for D-lactic acid and 1.24–4.23% for L-lactic acid. The R.S.D. of inter-day assays of peak areas of D- and L-lactic acids was from 3.14% to 6.50% and 0.54% to 6.00%, respectively; while those for migration time determination were from 0.59% to 2.66% and 0.59% to 2.77%, respectively.

3.4. Analysis of D- and L-lactic acids in plasma

In normal human plasma, the level of D-lactic acid is very small, lower than the detection quantities (LOQ). This work used acetonitrile for deproteinization of plasma samples, and then the supernatants were evaporated to concentrate D- and L-lactic acids.

Fig. 2 shows the electropherogram of a plasma sample from a short bowel syndrome patient. The peaks of L-lactic acid could be obviously observed, while these samples showed much smaller peaks of D-lactic acid, which could be completely separated from the responses of L-lactic acid. From the regression equations, the concentrations of L- and D-lactic acids in human plasma sample were determined to be 1.22 and 0.083 mM, respectively. After D-lactic acid were added to human plasma samples, their responses increased and the clear peaks of D-lactic acid were observed.

3.5. Comparison of the proposed CE method with the enzymatic assay

In order to evaluate the obtained values by the proposed CE method, D-lactic acid concentrations determined by the proposed CE method were compared with those by the enzymatic methods reported by Brandt et al. [16] using the

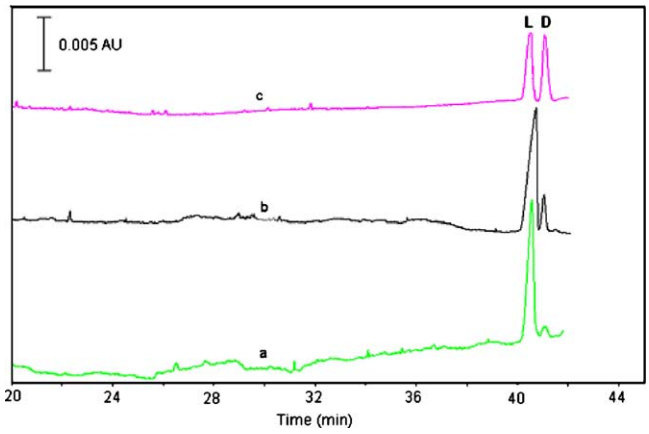

Fig. 2. Electropherograms of human plasma sample (a), the sample spiked with 0.5 mM D-lactic acid (b) and 10 mM D- and L-lactic acid mixture (c). Conditions: 150 mM phosphate–Tris buffer (pH 7.0) containing 220 mM 2HP-β-CD and 0.2 mM TTAB with an effective voltage of −25 kV at 18 °C.

same human plasma samples. Their correlation coefficient was 0.9622, indicating good agreement of the results of the two distinct methods. The enzymatic method needed more steps and larger volume of plasma sample (more than 1 ml), and could not simultaneously determine D- and L-lactic acids. Furthermore, it encountered the effect of cross-reactivity or the disturbance of enzymatic reaction by endogenous compounds. The proposed CE method overcame these disadvantages.

4. Conclusions

It is of great significance for clinical diagnosis to assay lactic acids, especially, D-lactic acid level in plasma. This work develops a new CE method with uncoated capillary to chirally separate L- and D-lactic acid enantiomers. Adding TTAB in the running buffer to reverse the electroosmotic flow and adjusting the pH of running buffer with Tris solution instead of sodium hydroxide can achieve desired resolution. In addition, the linearity and precision of this method are good. This proposed method has been applied to the determination of L- and D-lactic acid enantiomers in plasma samples by a protein-removal and a sample stacking procedure. This method is a rapid, simple and effective for clinical routine detection of L- and D-lactic acid enantiomers in plasma.

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