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SENSITIVE DETERMINATION OF D-LACTIC ACID IN BIOLOGICAL SAMPLES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

D-Lactate in biological samples was converted into the hydrazone of pyruvate in the presence of D-lactate dehydrogenase, an NADH-reoxidation system using diaphorase, DL-6,8-thioctamide and hydrazine. The hydrazone was converted into 2-methylquinoxanol by *o*-phenylenediamine in hydrochloric acid, and then the quinoxanol was determined by high-performance liquid chromatography with fluorescence detection. The calibration curve of D-lactate was linear up to at least 60 nmol/ml, and the determination limit was 600 fmol. Using this method, D-lactate was determined in biological samples.

INTRODUCTION

There is little information in the literature concerning the degradation and formation of D-lactate in animals, although considerable amounts of D-lactate are found in animal tissues and, furthermore, ruminants and human beings digest D-lactate. Ruminants absorb it in the stomach and humans consume it in yogurt and cheese. During a study of methylglyoxal [1,2], we aimed to study the biochemistry of D-lactate and so developed a sensitive determination method for D-lactate, which was applicable to biological samples.

EXPERIMENTAL

Chemicals

Lithium D-lactate was purchased from Sigma (St. Louis, MO, U.S.A.) and partially supplied by the kindness of Yakult (Tokyo, Japan). β-NAD^+, D-lactate dehydrogenase from *Leuconostoc mesenteroides* and diaphorase were purchased from Oriental Yeast (Tokyo, Japan). D-Lactate dehydrogenase from *Staphylococcus* sp. was kindly supplied by Amano Pharmaceutical (Nagoya, Japan). Hy-

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drazine sulphate and *o*-phenylenediamine (o-PDA) were obtained from Wako (Osaka, Japan). DL-6,8-Thioctamide was purchased from Tokyo Kasei Kogyo (Tokyo, Japan).

High-performance liquid chromatography

A Shimadzu LC6A liquid chromatograph was used with a Shimadzu RF-500LC fluorescence spectromonitor.

The separations of 2-methylquinoxanol (2-MQ) were performed on a Unisil ODS QT-5K column (150×4.6 mm I.D.) with isocratic elution using 10 m*M* potassium phosphate (pH 2.1)–acetonitrile (80:20) at a flow-rate of 1.0 ml/min. The excitation and emission wavelengths were 341 and 416 nm, respectively. 2-MQ can be also determined by UV absorption at 334 nm with a sensitivity 1/50 that of fluorescence detection.

Determination of D-lactate in water

The principle of the determination of D-lactate was as shown in Fig. 1. Various amounts of D-lactate (15–120 μl of 1 m*M* lithium D-lactate) were treated at 37°C for 2 h in a 5-ml vial with a tight fitting cap, and containing 50 μl of 10 m*M* NAD^+, 50 μl of D-LDH (100 U/ml), 100 μl of 26 m*M* DL-6,8-thioctamide, 50 μl of diaphorase (2.5 U/ml), 50 μl of 21 m*M* hydrazine sulphate and 0.1 *M* potassium phosphate (580–685 μl, pH 7.0). The hydrazone of pyruvate in the reaction mixture was converted into 2-MQ with 0.2 ml of 70 m*M* o-PDA in 1 *M* hydrochloric acid at 50°C for 1 h. The reaction mixture was brought to pH 4.0 with 2 *M* sodium phosphate and extracted with two 2-ml portions of ethyl acetate. The extraction yield was 92.0%. The ethyl acetate layer was evaporated using a Savant Speed Vac concentrator (Model SVC-100H, New York, NY, U.S.A.) at room temperature. The residue was dissolved in 0.4 ml of the mobile phase and analysed by HPLC (Fig. 2a). For the analysis of 2-MQ, the internal standard was not used, but 10 μl of 2-MQ (1 μg/ml of the mobile phase) were injected in each analysis for standardization and confirmation.

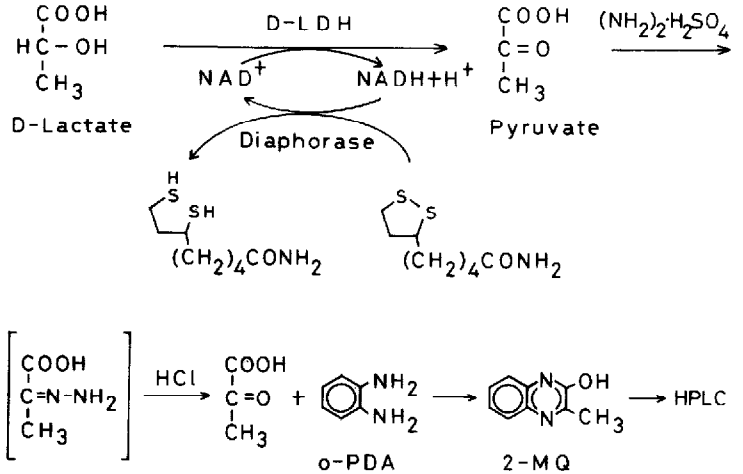

Fig. 1. Reaction scheme for the determination of D-lactate by HPLC.

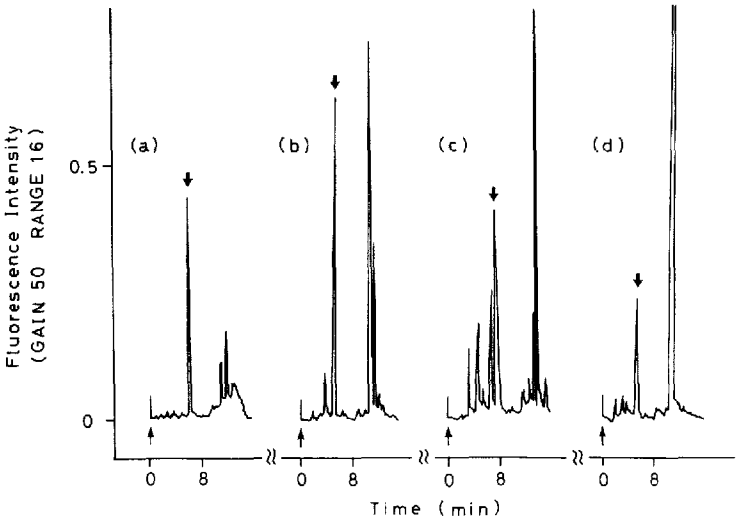

Fig. 2. High-performance liquid chromatograms. (a) Lithium D-lactate (60 nmol) was incubated at 37°C for 2 h in the presence of D-LDH, DL-6,8-thioctamide, diaphorase and hydrazine buffer. After incubation, the hydrazone was converted into 2-MQ with o-PDA, and 2-MQ was extracted with ethyl acetate. After evaporation, 2-MQ was dissolved in 0.4 ml of the mobile phase, 10 μl of which were injected. (b) Plasma (0.15 ml) was deproteinized with perchloric acid, and the supernatant was neutralized with potassium hydroxide. Thence it was treated as above. (c) Human urine (0.2 ml) was determined as in (a). An aliquot (10 μl) of dissolved 2-MQ (0.4 ml) was injected. (d) Rat liver was homogenized in 10 volumes of buffer. After centrifugation, the supernatant was deproteinized with methanol, followed by evaporation. Thereafter, it was treated as described in (a).

Determination of D-lactate and pyruvate in plasma

A plasma sample (0.15 ml) was deproteinized with two volumes of 0.5 *M* perchloric acid, and the supernatant was neutralized with 1 *M* potassium hydroxide, followed by a second centrifugation at 1700 *g* for 10 min. In a 5-ml vial with a tight cap, 0.5 ml of the supernatant, 50 μl of 21 m*M* hydrazine sulphate and 0.20 ml of 0.1 *M* potassium phosphate solution (pH 7.0) were mixed and allowed to react at 37°C for 30 min to eliminate endogenous pyruvate. The reaction was continued for a further 2 h at 37°C following the addition of 50 μl of 10 m*M* NAD^+, 50 μl of D-LDH (100 U/ml), 100 μl of 26 m*M* DL-6,8-thioctamide and 50 μl of diaphorase (25 U/ml). Then 0.3 ml of 1 *M* hydrochloric acid and 0.2 ml of 70 m*M* o-PDA were placed in the vial and allowed to react at 50°C for 1 h with shaking. The 2-MQ formed was treated as described for the determination of D-lactate in water (Fig. 2b). The blank test was carried out without addition of D-LDH and NADH-reoxidation system. The pyruvate present in plasma was determined as described above, except for the dehydrogenase reaction. The amount of D-lactate was obtained by subtracting that of the endogenous pyruvate.

Determination of D-lactate in human urine

Human urine samples (0.2 ml) were assayed for D-lactate as described for the determination of D-lactate in plasma, except for the deproteinization procedure.

For the analysis of 2-MQ by HPLC, 10 m*M* potassium dihydrogenphosphate (pH 2.1)–acetonitrile (81:19) was used as the mobile phase (Fig. 2c).

Preparation of rat liver samples and determination of D*-lactate in rat liver*

Male Wistar albino rats were decapitated, and the livers were removed immediately, washed and perfused with physiological saline. The livers were homogenized in ten volumes of 10 m*M* potassium phosphate (pH 7.0) using a Waring blender (at 10 000 rpm for 5 min). After centrifugation at 6000 *g* for 30 min, a 0.5-ml aliquot of the supernatant was placed in a 5-ml test-tube, and 0.5 ml of methanol was added. After centrifugation at 1700 *g* for 15 min, 1.5 ml of methanol were added to the supernatant and the mixture was allowed to stand for 5 h at −20°C. After further centrifugation at 1700 *g* for 15 min, the supernatant was transferred to the 5-ml vial and evaporated using the concentrator at room temperature. The dried residue was dissolved in 250 μl of 0.1 *M* potassium phosphate (pH 7.0), and 200 μl of resulting solution were treated as described for the determination of D-lactate in plasma (Fig. 2d).

RESULTS

Hydrazine sulphate as trapping agent

The equilibrium of the D-lactate dehydrogenase reaction by LDH of *Leu. mesenteroides* is in the favour of D-lactate ($K_{eq} = 4.5 \cdot 10^{-13}$ at 20°C). In order to force the reaction in the opposite direction, we first sought a trapping agent for pyruvate, such as o-PDA, sodium bisulphite, hydrazine, N,N-dimethylhydrazine, monomethylhydrazine, semicarbazide and hydroxylamine. They were tested in the incubation system using D-LDH, and hydrazine sulphate was found to be the best agent. In order to determine the effect of the hydrazine concentration on the dehydrogenase activity, D-lactate was treated at 30°C and 37°C for 2 h with D-LDH (1 U) and NAD^+ in 0.1 *M* of potassium phosphate buffer (pH 7.0) containing various amounts of hydrazine sulphate. After reaction, the hydrazone was converted into 2-MQ by o-PDA in 1 *M* hydrochloric acid and 2-MQ was determined by HPLC. The change of the molar ratio of hydrazine to D-lactate in the range 5–320 had no influence on the 2-MQ formation, and the yield of 2-MQ was 8% greater at 37°C than at 30°C. The hydrazone of pyruvate, which was treated with sodium pyruvate and hydrazine sulphate (1:1 molar ratio) at 37°C for 20 min in 1 *M* hydrochloric acid, showed one spot of R_F value 0.38 on silica gel TLC (*n*-butanol–acetic acid–water, 3:1:1), and neither pyruvate (R_F 0.48) nor hydrazine (R_F 0.58) was found. After pyruvate (30–240 nmol) was treated with hydrazine (9.6 μmol) in 0.1 *M* potassium phosphate (pH 7.0) at 37°C for 50 min, the hydrazone formed was further treated with o-PDA in 1 *M* hydrochloric acid and treated as above. A 20-μl aliquot of 1 ml of the dissolved residue was analysed by HPLC. A plot of *y* for 2-MQ vs. *x* for pyruvate gave a straight line. This means that pyruvate can be determined in the range 0.6–4.8 nmol/20 μl, and the hydrazone formed was quantitatively converted into 2-MQ by calculating from the standard curve. The yield of 2-MQ was 87.5%.

Optimum conditions for formation of 2-MQ

In order to determine the optimum reaction temperature, time and molar ratio of o-PDA to pyruvate, 0.5 ml of 1 m*M* pyruvate and 0.5 ml of 1 m*M* hydrazine sulphate were placed in a 5-ml vial and allowed to react at 37°C for 30 min. The o-PDA solution in 1 *M* hydrochloric acid was successively added to the reaction mixture by changing the reaction time, temperature or molar ratio of o-PDA to pyruvate. It became apparent that the yield of 2-MQ was optimal between 30 and 60 min at 50°C, between 50 and 100°C for 30 min, and reached a maximum above a molar ratio of 20 (o-PDA to pyruvate) at 50°C for 30 min. Pyruvate and o-PDA were treated with 1 *M*, 2 *M* and 6 *M* hydrochloric acid, as well as with 0.5 *M* perchloric acid, and 2-MQ was obtained in the best yield with 1 *M* hydrochloric acid.

Stability of 2-MQ

When 2-MQ was dissolved in the mobile phase and allowed to stand at room temperature for 24 h in a test-tube covered with parafilm, no chemical change was observed. 2-MQ was also stable on heating at 27°C, 50°C and 100°C in 6 *M* hydrochloric acid for 1 h in a vial with a cap.

Calibration curve

Adding hydrazine as a trapping agent to the dehydrogenase reaction system resulted in a non-linear calibration curve. When the NADH-reoxidation system was also added to the system, the calibration curve was linear up to at least 60 nmol/ml. As little as 250 pmol/ml of D-lactate could be measured by the present procedure.

Recovery test

Various amounts of D-lactate were added to the supernatants of rat liver homogenate and determined as described under Experimental. The results, shown in Table I, indicate ca. 80% recovery. Table I also shows the recoveries from human plasma and urine. These values were greater than those from the supernatants of liver homogenate. The recovery from a fermented milk drink (Calpis) was 85.1%.

D-Lactate in yogurt

Three kinds of fermented milk, which were bought in a nearby market, were analysed for D-lactate. They were centrifuged at 10 000 *g* for 30 min, and the supernatant was analysed for D-lactate. Yogurt (LB51) made by Meiji Milk Products contains 6.73 ± 0.62 μmol/g wet weight; Calpis drink (Calpis Food Industry), 17.81 ± 1.20 μmol/ml; Yakult drink (Yakult), 0.24 ± 0.03 μmol/ml. After analysis we found out that Yakult drink was fermented by bacteria producing L-lactate.

D-Lactate in human plasma

Blood samples were collected from the cubital vein of normal human adults before and at 1.5 h after lunch, and analysed for D-lactate and pyruvate. The

TABLE I

RECOVERY OF D-LACTATE

After various amounts of D-lactate were added to the supernatants of rat liver homogenate, deproteinized human plasma and urine, D-lactate was determined as described in Experimental.

D-Lactate added	D-Lactate recovered	Recovery (%)
Rat liver homogenate (nmol/0.1 g wet weight)		
0	10.7	
15	23.1	82.9
30	35.4	82.2
60	56.4	76.2
90	78.9	75.8
	Mean ± S.D.	79.3 ± 3.3
Deproteinized human plasma (nmol/ml plasma)		
0	20.6	
14	34.4	89.1
28	46.1	90.4
56	67.9	84.1
84	89.4	81.2
	Mean ± S.D.	86.2 ± 4.3
Deproteinized human urine (nmol/0.5 ml urine)		
0	29.9	
15	43.6	91.6
30	61.0	103.7
60	83.8	89.9
90	118.8	98.8
	Mean ± S.D.	96.0 ± 5.6

TABLE II

DETERMINATION OF D-LACTATE AND PYRUVATE IN HUMAN PLASMA BEFORE AND AFTER LUNCH

Blood samples were collected from the cubital vein of our collaborators before and after lunch, and analysed for D-lactate and pyruvate as described in the text. Concentrations are in nmol/ml plasma.

Subject	Age	D-Lactate			Pyruvate	
		Before	After	Increase (%)	Before	After
K.N.	26	12.7	42.5	335	39.1	22.5
T.B.	23	36.9	82.1	223	20.2	30.3
Y.T.	23	29.3	71.3	243	35.7	25.2
T.I.	24	33.3	68.5	206	14.8	19.2

lunch in our university cafetaria consisted roughly of 200 g of rice, 150 g of fried fish, hamburger, 130 g of potato salad and 100 ml of miso-soup. These results are listed in Table II. D-Lactate concentration more than doubled, while the pyruvate

TABLE III

DETERMINATION OF D-LACTATE AND PYRUVATE IN HUMAN PLASMA BEFORE AND AFTER SHORT-TIME RUNNING

Students and collaborators ran for ca. 5 min. Blood samples were obtained from the cubital vein at rest and after running. D-Lactate and pyruvate were determined as described in the text.

Subject	Age	D-lactate			Pyruvate		
		Before (nmol/ml)	After (nmol/ml)	Increase (%)	Before (nmol/ml)	After (nmol/ml)	Increase (%)
N.K.	26	21.8	68.2	313	21.8	80.5	369
T.B.	23	17.4	58.5	336	11.9	54.7	460
Y.T.	23	28.2	91.5	324	24.7	70.6	286
T.I.	24	19.7	42.8	217	23.0	68.9	300
T.O.	25	43.0	71.5	166	29.6	94.4	319
M.I.	36	47.6	53.2	112	26.4	49.3	187

TABLE IV

D-LACTATE AND PYRUVATE LEVELS IN PLASMA OF DIABETIC RATS

Rats aged 4 weeks were injected intraperitoneally with streptozotocin (75 mg/kg) and received normal solid food and water for 17 days. On the 17th day they were killed by decapitation and blood samples were collected, which were treated as described in the text. Values are mean ± S.D.

	Control ($n=5$)	Streptozotocin diabetic ($n=6$)	$P<0.05$
Body mass (g)	228.0 ± 23.9	178.3 ± 17.2	
Blood glucose (mg/dl)	110.0 ± 3.54	615.8 ± 79.3	
D-Lactate (nmol/ml)	21.6 ± 13.8	61.3 ± 16.0	↑
Pyruvate (nmol/ml)	38.4 ± 18.2	37.3 ± 9.92	–

level increased or decreased. We also examined D-lactate concentrations in blood after exercise. Students and co-workers in our laboratory ran five times up and down ten stairs of a five-floor building as fast as possible for ca. 5 min. Blood samples were immediately collected, and the plasma separated. Table III gives the concentrations of D-lactate and pyruvate. It was very interesting that even after short-time exercise D-lactate and pyruvate levels were markedly elevated.

D-Lactate level in blood of diabetic rats

Male Wistar strain albino rats aged 4 weeks were used. The rats were divided into two groups. One group weighing 89.2 ± 1.9 g was injected once intraperitoneally with streptozotocin (75 mg/kg). Another group (80.0 ± 4.5 g) was given the same volume of physiological saline. They received normal solid food and water. The rats were killed by decapitation on the 17th day after injection and blood samples were collected in a heparinized vessel. After blood was centrifuged,

plasma was tested for D-lactate and pyruvate. As shown in Table IV, the D-lactate concentration in blood increased markedly, while the pyruvate level remained unaltered.

DISCUSSION

D-Lactate has been determined by the extinction change at 340 nm [3–5], the automated micro method [6] and gas chromatography [7]. These methods are much less sensitive than the method presented here, which has detection limits of 31.1 pmol by UV detection at 334 nm and 600 fmol by fluorescence detection The present method is based on two devices; one is the use of a trapping agent and the reoxidation system, resulting in linear calibration curves, and the other is the conversion of D-lactate into a stable fluorescent derivative.

The steer plasma D-lactate concentration has previously been reported to be 0.38 m*M* [8]. This concentration is an order of magnitude higher than that of the human adult, whereas that in rat plasma is almost the same as in a healthy human adult [9,10]. This is not surprising, because it is known that ruminants absorb D-lactate from their own stomach, in which it is fermented. Conner et al. did not detect D-lactate in human blood [11]. These data must be due to an experimental error, because we always found a certain level of D-lactate in blood.

Urinary D-lactate excretion of a normal subject was reported to be 0.1–0.8 μmol/h [11]. This value is almost the same as our results. It is interesting to note that the D-lactate level in blood rises after exercise and eating, and in diabetics. Haralambie and Mössinger also reported that, after exercise, the D-lactate concentration in blood rose by 70%; they assumed this occurred via the aminoacetone pathway [12]. If D-lactate was formed from pyruvate in animals, the concentration of D-lactate would be expected to parallel that of pyruvate, but our data showed that two concentrations were altered independently. These problems are under study. In particular, we have been investigating the formation in rats of methylglyoxal, which is a precursor of D-lactate.

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