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Fluorimetric and high-performance liquid chromatographic determination of D-lactate in biological samples

SHINJI OHMORI*, YUKO NOSE, HIROKO OGAWA, KOHICHI TSUYAMA and TAKASHI HIROTA

Faculty of Pharmaceutical Sciences, Okayama University, Tsushima-Naka-1-1, Okayama 700 (Japan)

HIROSHI GOTO

Nissho Co. Nojicho, Kusatsu-city, Shiga 525 (Japan)

and

YOSHIKO YANO, YASUNORI KONDOH, KUNIHIKO NAKATA and SEIJI TSUBOI

Faculty of Pharmaceutical Sciences, Okayama University, Tsushima-Naka-1-1, Okayama 700 (Japan)

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ABSTRACT

D-Lactate in biological samples was converted into a strongly fluorescent substance in a one-vial reaction. It was first converted into the pyruvate hydrazone in the presence of D-lactate dehydrogenase, an NADH-reoxidation system using diaphorase, D,L-6,8-thioctamide and hydrazine. This hydrazone was then converted into 2-hydroxy-6,7-dimethoxy-3-methylquinoxaline by 1,2-diamino-4,5-dimethoxybenzene in 1 *M* hydrochloric acid, and the quinoxaline was extracted and measured fluorimetrically at 432 nm (excitation at 365 nm). The calibration curve for D-lactate was linear up to at least 100 nmol/ml of the assay mixture, with a determination limit of 2 nmol/ml. The quinoxaline was also analysed by high-performance liquid chromatography with fluorimetric detection. The calibration curve for D-lactate was linear from 500 fmol to 75 nmol in the reaction mixture. This method was 4000 times more sensitive than the fluorimetric method, and could determine D-lactate in blood plasma volumes of less than 1 μl.

INTRODUCTION

To study the metabolism of the methylglyoxal bypass of mammals, we established an assay for D-lactate [1]. During the purification of D-lactate dehydrogenase from rat muscle and yeast, a method was required by which a large number of samples could be assayed in a short time. For this purpose we developed a sensitive determination of D-lactate in biological samples. This method depends on the formation of 2-hydroxy-6,7-dimethoxy-3-methylquinoxaline (HDMQ) from pyruvate and 1,2-diamino-4,5-dimethoxybenzene (DADMB). However, DADMB was expensive and, more importantly, impure. Fortunately, the pure diamine could be simply and readily prepared, and as a result the method was developed

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as described below. Furthermore, the HDMQ formed was analysed by high-performance liquid chromatography (HPLC) with spectrofluorimetric detection.

EXPERIMENTAL

Chemicals

Lithium D-lactate was purchased from Sigma (St. Louis, MO, U.S.A.). D-Lactate dehydrogenase (D-LDH, EC 1.1.1.28) from *Staphylococcus sp.* and diaphorase (EC 1.6.4.3) from *Clostridium kluiveri* were kindly supplied by Amano Pharmaceutical (Nagoya, Japan). β-NAD^+ and its specially pure grade were also kind gifts from Oriental Yeast (Tokyo, Japan). D,L-6,8-Thioctamide was from Tokyo Kasei Kogyo (Tokyo, Japan), hydrazine sulphate and sodium pyruvate from Wako (Osaka, Japan) and pyruvic acid and veratorole from Katayama Chemical (Osaka, Japan). The quality of sodium pyruvate must be checked just before use, since it seems to decompose slowly with time.

Preparation of DADMB

4,5-Dinitroveratrole was synthesized from veratorole [2,3]. Fresh sodium hydrosulphite (80 g) and sodium hydroxide (10 g) were dissolved in 500 ml of water in a 2-l three-necked flask with a reflux condenser, a dropping funnel and a nitrogen gas inlet. Dinitroveratrole (11.4 g) in a mixture of 300 ml of ethanol and 100 ml of dioxane, was added dropwise for 40 min to the flask at 70°C under bubbling of nitrogen. The reaction was allowed to proceed for 70 min, the mixture was cooled at room temperature, and the precipitate was filtered. The filtrate was concentrated to *ca.* 100 ml under reduced pressure. After the concentrate had been extracted with chloroform (three 100-ml volumes), the chloroform extracts were combined and reextracted with 3 *M* HCl (two 100-ml volumes). The extract was evaporated under reduced pressure, and the residue was recrystallized from ethanol, giving white long needles [9.5 g, 79%, m.p. 230°C (decomposition)]. When the needles were recrystallized from ethanol, the same platelets were obtained. The R_F values of both lots of crystals were 0.25 on thin-layer chromatography (silica gel, ethyl acetate–acetic acid, 20:1, v/v). Calculated for $C_8H_{12}N_2O_2 \cdot 2HCl$ (needles): C, 39.85; H, 5.85; N, 11.62. Found: C, 39.74; H, 5.93; N, 11.64. Calculated for $C_8H_{12}N_2O_2 \cdot HCl$ (platelets): C, 46.94; H, 6.36; N, 13.69. Found: C, 46.87; H, 6.40; N; 13.84. The mass spectra of the two types of crystal showed the same fragmentation pattern. The parent peak of free base appeared at *m/z* 168, and considerable fragmentation peaks at *m/z* 153 ($M^+ - CH_3$) and 125 ($M^+ - CH_3 - CO$) were observed. IR (KBr) (needles): 3360, 2890, 2540 (N^+H_3) cm^{-1}; NMR ([$^2H_6$]DMSO): 3.75 (6H, singlet, 2 × OCH_3), 7.02 (2H, singlet, benzene-H), 8.50 (6H, broad, 2 × N^+H_3, disappeared with ^2H_2O addition).

When dinitroveratrole (1 g) and sodium hydrosulphite (8 g) were refluxed in 200 ml of methanol for 1 h, an orange reaction mixture was obtained. After cooling, the inorganic precipitate was filtered and washed with ethanol. The fil-

trate and washings were evaporated under reduced pressure. The residue was dissolved in 50 ml of 1 *M* sodium hydroxide containing 500 mg of sodium hydrosulphite, and extracted three times with diethyl ether. After evaporation of diethyl ether, the residue was recrystallized from methanol. Orange needles of N-(4,5-dimethoxy-2-nitrosophenyl)hydroxylamine were obtained. The R_F value was 0.23 on TLC (silica gel, $CHCl_3$); m.p. 167–171°C (closed capillary). IR (KBr): 3430, 3310 (O–H, N–H), 1495 (N=O) cm^{-1}; MS *m/z*: 198 (M^+), 183 ($M^+ - CH_3$), 168 ($M^+ - 2 \times CH_3$), 152 ($M^+ - CH_3 - HNO$), 137 ($M^+ - 2 \times CH_3 - HNO$); NMR ($C^2HCl_3$ δ): 3.86, 3.91 (each 3H, singlet, $2 \times OCH_3$), 6.17 (3H, singlet with broad absorption at δ 5.8–6.5 and the signal changed to one-proton singlet with ^2H_2O addition, 6-H, NH, OH), 7.54 (1H, singlet, 3-H). Calculated for $C_8H_{10}N_2O_4$: C, 48.48; H, 5.09; N, 14.14. Found: C, 48.21; H, 5.09; N, 14.09. HDMQ was prepared from the equivalent weight of DADMB monohydrochloride and sodium pyruvate. Both compounds were dissolved in 2 *M* HCl and allowed to stand at 20°C for 24 h. The crystals, when filtered and recrystallized from water, were pink needles, m.p. 258 °C (255°C in ref. 4), soluble in chloroform or chloroform–methanol (9:1, v/v), slightly soluble in methanol, and practically insoluble in benzene, diethyl ether and ethyl acetate.

Instrumentation

HDMQ was measured with an RF-500 LC fluorescence spectrophotometer from Shimadzu (Kyoto, Japan). For the HPLC determination of D-lactate with fluorimetric detection, a Model LC-6A (Shimadzu) liquid chromatograph was used, equipped with a 150 mm × 4.6 mm I.D. column with Wakosil $5C_{18}$ (Wako, Osaka, Japan) and a Shimadzu RF-535 fluorescence detector set to an excitation wavelength of 362 nm and an emission wavelength of 445 nm. The mobile phase was 10 m*M* dipotassium hydrogenphosphate (pH 6.0 adjusted with 85% phosphoric acid)–acetonitrile (80:20, v/v). The flow-rate was 1.0 ml/min and all runs were performed at 40°C.

Determination of D-lactate in water

The principle of the determination of D-lactate is shown in Fig. 1. Various amounts of D-lactate (10–40 nmol) were incubated with 100 µl of 20 m*M* hydrazine sulphate, D-LDH (100 U/ml), 25 m*M* thioctamide, 50 µl of 10 m*M* NAD^+ and diaphorase (95 U/ml) in total volume of 1 ml by adding 0.1 *M* potassium buffer (pH 7.0) at 37°C for 2 h. The incubation was run in a 5-ml vial with a tightly fitting cap. After incubation, 500 µl of 5 m*M* DADMB in 3 *M* HCl was added to the vial and nitrogen gas was flushed before capping. The vial was heated at 80°C for 1 h. The reaction mixture was adjusted to pH 2.0 with 0.8 ml of 2 *M* K_3PO_4, and extracted twice with 2 ml of chloroform. The extracts (1.5 ml) were combined, and the fluorescence was measured at 432 nm following excitation at 365 nm. For the HPLC analysis the extract was evaporated under reduced pressure, and the residue was dissolved in 1 ml of the mobile phase, of which 10 µl were injected into the chromatograph.

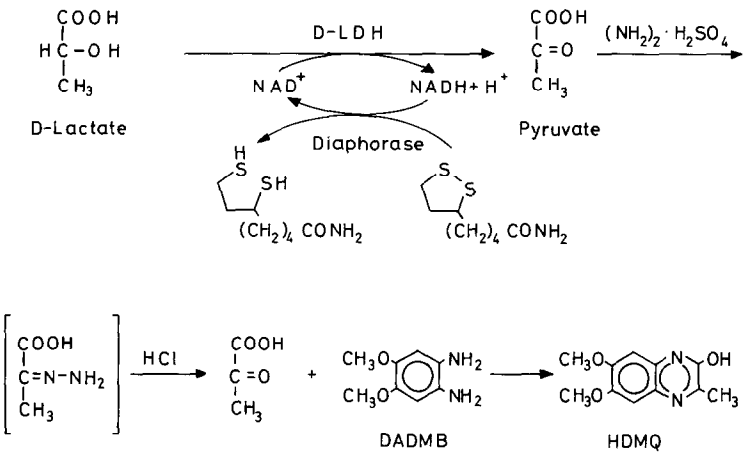

Fig. 1. Reactions involved in the determination of D-lactate.

Determination of D-lactate in rat liver

Fresh rat liver was perfused with physiological saline and homogenized in 10 volumes of 10 m*M* potassium phosphate buffer (pH 7.0) using a Waring blender. The homogenate was centrifuged at 6000 *g* for 30 min. The supernatant (0.4 ml) was mixed with 1.6 ml of methanol and centrifuged at 1700 *g* for 15 min. The supernatant was transferred to a 10-ml test-tube, and the precipitate was homogenized with 1.2 ml of methanol–water (70:30, v/v), followed by the same centrifugation step. The methanol extracts were combined and kept in a freezer (−20°C) for 3 h, then centrifuged as described above the remove the precipitate. The supernatant was transferred to a 5-ml vial and evaporated using a Savant Vac concentrator (Model SVC-100H, New York, NY, U.S.A.) at room temperature. After the residue had been dissolved in 0.6 ml of 0.1 *M* potassium phosphate (pH 7.0), 0.1 ml of 20 m*M* hydrazine sulphate was added to the vial. The reaction proceeded at 37°C for 30 min, to prevent the endogenous pyruvate from undergoing the reverse reaction. It was treated for a further 2 h at 37°C after the addition of 50 μl of 10 m*M* NAD^+, 100 μl of D-LDH (100 U/ml), 100 μl of 25 m*M* D,L-6,8-thioctamide and 50 μl of diaphorase (95 U/min). The trapped pyruvate was converted into HDMQ, which was extracted with chloroform and measured fluorimetrically as described for the determination of D-lactate in water. As a blank test sample, 1 ml of 0.1 *M* potassium phosphate (pH 7.0) was added to the solution of trapped pyruvate instead of the D-LDH and NADH-reoxidation system. For HPLC analysis the chloroform layer was evaporated under reduced pressure using the concentrator, and the residue was dissolved in 1 ml of the mobile phase, of which 10 μl were injected into the chromatograph.

Determination of D-lactate in human blood plasma

A plasma sample (0.2 ml) from heparinized human blood was deproteinized

with 2 ml of methanol and centrifuged at 1700 *g* for 15 min. A 1.5-ml volume of 70% methanol (methanol–water, 70:30 v/v) was added to the precipitate, followed by the same centrifugation step. The combined supernatant was treated as described for the determination of D-lactate in rat liver. HPLC analysis of D-lactate was carried out as described for liver samples.

RESULTS

Reaction conditions for the formation of HDMQ from pyruvate

To determine D-lactate, it was converted into pyruvate, then treated with DADMB to form HDMQ. In a series of reactions, it turned out that NAD^+ and DADMB gave some fluorescent product, if the reaction temperature was higher, regardless of the purity of NAD^+. To prevent the formation of the fluorescent product from NAD^+, pyruvate formed from D-lactate was allowed to react with DADMB at 80°C for 1 h. Experiments with various concentrations of HCl revealed that the conversion of pyruvate into HDMQ should be carried out in 1 *M* HCl. A molar ratio of DADMB to pyruvate of *ca.* 20 produced the best yield of the quinoxalinol under the reaction conditions described. The yield of HDMQ from pyruvate was 98.6 ± 2.0% ($n=4$) as measured by the HPLC method.

Optimum determination conditions for D-lactate

Using "the determination method of lactate in liver", optimal amounts of diaphorase, D-LDH, NAD^+, hydrazine and thioctamide were defined to be 5 U, 10 U, 0.5 μmol, 2 μmol and 2.5 μmol, respectively. However, the concentration of each cofactor and enzyme other than D-LDH did not exert a significant influence on the formation of HDMQ, as was to be expected.

Extraction of HDMQ

Because HDMQ has an amphoteric character, the pH of the extraction medium was examined. To 4 ml of chloroform solution of HDMQ (10 nmol) were added 4 ml of 1 *M* HCl, H_3PO_4, $NaHCO_3$ and Na_2CO_3, respectively; the mixture was mixed well. The chloroform layers were determined fluorimetrically, with recoveries of 53.5, 92.1, 97.1 and 17.0%. The extraction efficiencies with chloroform were 76.0 and 90.6% for HDMQ in 0.5 and 0.1 *M* HCl, respectively. From these results, it was possible to extract HDMQ under weakly alkaline or acidic conditions. However, when fluorimetry is used, it is desirable that HDMQ is extracted under acidic conditions, because of elimination of DADMB and prevention of turbidity.

Fluorescence intensity and stability of HDMQ

The relative fluorescence intensities of HDMQ, 2-hydroxy-3-methylquinoxaline (HMQ) and 2-methylquinoxaline in chloroform were 4100, 4.1 and 1, respectively, at 432 nm following excitation at 365 nm. Their relative intensities in the

mobile phase (pH 6.0) were 3500:40:1, which were measured at 445 nm (excitation at 362 nm), 422 nm (excitation at 333 nm) and 426 nm (excitation at 316 nm), respectively.

The relative fluorescence intensity of HMDQ varied with the pH: it was 1, 1300 and 1670 at pH 2.1, 6 and 7.5, respectively. The pH 6 of the mobile phase was chosen because of pH allowance of the column. HDMQ had a very high fluorescence intensity compared with HMQ, which was used in the previous study.

No degradation was observed by HPLC when HDMQ was dissolved in chloroform or the mobile phase (pH 2.1 and 6.0), and allowed to stand at room temperature for 12 days or at 4°C for 90 days in the dark.

High-performance liquid chromatograms of HDMQ derived from D-*lactate.*

Fig. 2a shows the elution profile obtained when lithium D-lactate solution (5 nmol) was dehydrogenated by D-LDH, and the pyruvate formed was converted into HDMQ, which was extracted with chloroform and assayed by HPLC with fluorimetric detection, as described under *Determination of* D-*lactate in water.*

The "target" shows the peak of HDMQ at 5.0 min. Three other peaks, at 1.5,

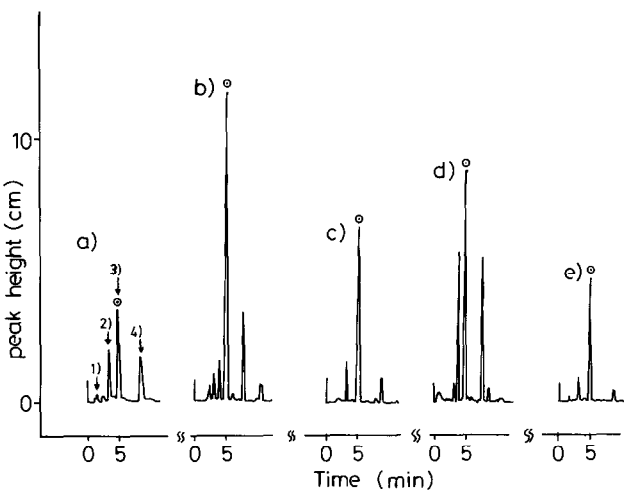

Fig. 2. High-performance liquid chromatograms. (a) Lithium D-lactate (10 nmol) was incubated at 37°C for 2 h in the presence of D-LDH, D,L-thioctamide, diaphorase and hydrazine buffer. After incubation, the hydrazone was converted into HDMQ with DADMB, and HDMQ was extracted with chloroform. After evaporation, HDMQ was dissolved in 1 ml of mobile phase, and 10 μl of the solution were chromatographed. (b) Rat liver was homogenized in 10 volumes of buffer, and the supernatant (0.4 ml) was deproteinized with methanol, followed by evaporation. Thereafter, it was treated as described in (a). Intrinsic pyruvate and pyruvate from D-lactate appear as one peak. (c) The deproteinized sample from rat liver was treated without D-LDH or the NADH-reoxidation system. The remaining peak is due to intrinsic pyruvate. (d) Plasma (0.2 ml) was deproteinized with methanol, and thereafter treated as in (b). (e) Plasma sample was treated without D-LDH or the NADH-reoxidation system, as a blank.

3.3 and 8.3 min, may be due to the reagents. Since DADMB is unstable to light and oxygen, care must be taken to prevent the appearence of concomitant peaks.

The HPLC analyses of rat liver are illustrated in Fig. 2b and c. Rat liver (1 g) was homogenized in 10 volumes of buffer. The homogenate was centrifuged and the supernatant (0.4 ml) was deproteinized with methanol. The deproteinized solution was evaporated under reduced pressure, and the residue was analysed for intrinsic pyruvate and D-lactate (Fig. 2b). As a blank test sample, the residue from 0.4 ml of the supernatant was not treated without D-LDH and the NADH-reoxidation system and analysed directly for intrinsic pyruvate (Fig. 2c).

The method was also applied to human blood plasma (0.2 ml). When deproteinized plasma is treated with D-LDH and the reoxidation system, both pyruvate in plasma and pyruvate from D-lactate in plasma are analysed (Fig. 2d), whereas only intrinsic pyruvate is analysed without D-LDH as the blank (Fig. 2e). The amount of D-lactate was obtained by subtracting the blank value. This method has the advantage that pyruvate is determined at the same time.

Calibration curves and limits of determination

Various amounts (10–40 nmol) of D-lactate were incubated in 1 ml of the assay mixture and treated as described under *Determination of D-lactate in water*. In the case of fluorimetric determination the fluorescence intensity (y, gain 5) was directly proportional to the D-lactate concentration (x) in the assay mixture over the range 2–100 nmol/ml ($n=6$); $y = 0.5740x + 0.5142$ ($r = 0.9997$). The determination limit was 2 nmol, and the detection limit of HDMQ in chloroform was 0.5 pmol/ml. For the HPLC method, calibration curves were as follows. A plot of the peak height (y) of the fluorescence intensity *versus* the D-lactate concentration (x) in the assay mixture gave a straight line from 5 to 75 nmol/ml ($n=5$, range 128), y (cm) $= 0.1994x - 0.4202$ ($r=0.9993$); from 25 to 200 pmol/ml ($n=5$, range 32), y (mm) $= 0.4202x - 0.5256$ ($r=0.9986$); from 0.5 to 20 pmol/ml ($n=6$, range 16), y (cm) $= 0.8017x - 0.0575$ ($r=0.9999$). From these curves, the determination limit was found to be 0.5 pmol/ml, indicating that the HPLC method is 4000 times more sensitive than the fluorimetric method presented here. The detection limit of HDMQ was 0.4 fmol/10 μl by the HPLC method.

Recovery test

Various amounts (1, 2, 3 and 5 nmol) of D-lactate were added to 0.2 ml of human blood plasma and determined as described under *Determination of D-lactate in human blood plasma*. The recovery was 95.3 $\pm$ 5.6% for the HPLC method. The recoveries of D-lactate from 0.4 ml of the supernatant of deproteinized rat liver homogenate were determined by the HPLC method. The recovery of added amounts in the range 1–5 nmol was 79.6 $\pm$ 3.5% ($n=5$). Similar recoveries were obtained with the fluorimetric method.

DISCUSSION

In 1985 Hara and co-workers [5,6] reported a fluorimetric method for the determination of α-keto acids using DADMB. At that time we attempted to prepare DADMB according to their method, but we failed to obtain pure DADMB in a good yield. We made further efforts to make DADMB, and as a result we were able to prepare it in pure form in a good yield. Consequently, D-lactate in biological samples could be determined using DADMB. During the study of its preparation, N-(4,5-dimethoxy-2-nitrosophenyl)hydroxylamine was obtained according to selection of the reaction solvent, in which, however, no DADMB was obtained.

We have reported previously a method for the determination of D-lactate, in which D-lactate was converted into HMQ by *o*-phenylenediamine, which was determined by HPLC with fluorescence detection. Using this method, D-lactate was determined up to at least 60 nmol/ml, and as little as 250 pmol/ml D-lactate in the assay mixture could be measured. The detection limit of HMQ was 600 fmol per 10-μl injection. It was found the relative fluorescence intensity of HMQ also varied with the pH, and the method was improved by using a mobile phase of pH 6.0. This increased the sensitivity by over twenty-fold. Since HDMQ is much more strongly fluorescent than HMQ, the fluorimetric method described here can determine 2–100 nmol of D-lactate, but the HPLC method can determine D-lactate in the range from 500 fmol to 75 nmol. D-Lactate in less than 1 μl of blood plasma can be measured by the HPLC method. Since both methods can determine small amounts of D-lactate, our studies of the methylglyoxal bypass in animals will be enhanced.

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