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Determination of pyruvate and lactate in primary liver cell culture medium during hypoxia by on-line microdialysis–liquid chromatography

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Abstract

A microdialysis sampling device was constructed for the measurement of pyruvate and lactate in primary liver cell culture medium during hypoxia. It was composed of a Petri dish, a dialysis membrane and two transmission tubes within a hypoxia chamber. The dialysis membrane was located in the Petri dish such that it was immersed in the culture medium. Dialysates were collected and introduced by an on-line injector to a liquid chromatographic system for analysis of pyruvate and lactate. The detection limit of this assay was 0.2–2.0 μ*M* with acceptable intra- and inter-assay reproducibilities. In order to validate the assay, primary liver cells were incubated in the Petri dish within a hypoxia chamber in an incubator. The baseline concentrations of pyruvate and lactate in primary liver cell culture medium were 10.6±5.6 and 607±143 μ*M*, respectively. These levels drastically changed during hypoxia and reperfusion. In conclusion, the present assay provides a sensitive, direct measurement of pyruvate and lactate in culture medium while minimizing pretreatment procedures for sample preparation.
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Keywords: Microdialysis; Pyruvate; Lactate; Organic acids

1. Introduction

Microdialysis is one of the most widely used techniques for in vivo and in vitro sampling of the chemical substances in extracellular fluids of animal tissues or cultured cells [1,2]. The measurement of pyruvate and lactate utilizing LC–UV is useful in investigating the pathology of energy crises in animal models or cell cultures [3,4].

Glucose is formed via carbohydrate digestion and conversion of glycogen to glucose by the liver [5]. The energy needs of liver cells are supplied by aerobic metabolism of glucose and oxygen in the mitochondrial respiratory chain. Lactate and pyruvate are representative products of carbohydrate metabolism under anaerobic and aerobic conditions, respectively. The evolution of reversible to irreversible damage in liver cells involves progressive derangement in energy and substrate metabolism. Large amounts of lactate can be produced during periods of anaerobic metabolism when cells do not receive adequate oxygen. Under these conditions, pyruvate is reduced to lactate by lactate dehydrogenase. During ischemia or hypoxia, small amounts of adenosine triphosphate (ATP) can be produced by anaerobic glycolysis. Therefore, the glycogen stores

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are depleted after the onset of ischemia. Glycogen is metabolized to lactate which causes progressive intracellular acidosis. Changes in lactate and pyruvate have been used as important biochemical markers of ischemia in experimental animals and clinical studies [6–8].

Microdialysis and LC–UV are complementary tools that are routinely used in this laboratory to study energy-related metabolites in animal models and cultured cells [4,9,10]. In the present study, a technique for the direct measurement of pyruvate and lactate in primary liver cell culture medium by microdialysis was developed. This novel sampling device was constructed within a hypoxia chamber. Dynamic changes in pyruvate and lactate levels in primary liver cell culture medium under hypoxia and reperfusion were demonstrated.

2. Experimental

Primary liver cells were isolated from male Sprague-Dawley rats weighing 200–250 g by two-step collagenase perfusion [11]. Rats were anaesthetized with sodium pentobarbital (100 mg/kg, i.p.), then the liver was perfused via the portal vein at a flow-rate of 25 ml/min with 150 ml of HBSS buffer to remove any blood. The liver was perfused again at a flow-rate of 10 ml/min with 100 ml of the same buffer supplemented with 40 mg collagenase. Then, the liver was removed, sieved through a steel mesh, washed, and suspended in 100 ml of William E buffer. The suspension was centrifuged (100 *g*, 15 min) to isolate hepatic parenchymal cells. The hepatic parenchymal cells were washed with Percoll buffer (10% Hank's Balanced Salt Solution (HBSS) buffer and 90% Percoll) and centrifuged again (100 *g*, 15 min). Cell pellets were re-suspended with Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Gaithersburg, MD, USA) plus 5% horse serum (HS) and 10% fetal bovine serum (FBS). Cell viability was determined by the trepan blue exclusion method (and was typically greater than 90%). Primary hepatocytes were plated onto NCNC T75 flasks (Naperville, IL, USA) with DMEM plus 5% HS and 10% FBS, and maintained in a 10% CO_2 humidified incubator at 37°C, as previously described [12]. The hepatocytes were re-plated onto a 3.5 cm Petri dish at a density of $3\cdot10^5$ cells/dish in a medium with DMEM plus 5% HS and 10% FBS and incubated in a 37°C, 5% CO_2 incubator. Later, the medium was changed with William E (containing 10% FBS serum) and the cells were cultured overnight. Primary liver cells were cultured on a 35 mm Petri dish with DMEM. The working buffer was prepared by dissolving 0.17 g 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) in 25 ml liquid medium of DMEM base, which contained L-glutamine (584 mg/l), phenol red (15 mg/l), and sodium pyruvate (110 mg/l). The pH of the buffer was then adjusted to 7.4.

Standard stock solutions of pyruvate and lactate were prepared at concentrations of 10 and 100 m*M*, respectively, in 4 m*M* sulfonic acid, and stored at 4°C. The standard mixtures were prepared from a portion of these stock solutions after appropriate dilution with 4 m*M* sulfonic acid. In vitro recovery was performed in a standard mixture containing pyruvate and lactate to determine the recoveries of all analytes and the dead volume of the microdialysis sampling device. The sampling device was composed of a Petri dish, two transmission tubes, and a dialysis tube within a hypoxia chamber. The Petri dish had a receiving space with an open top. The cover contained two holes separated by a predetermined distance. The top ends of the two transmission tubes jutted out through the holes in the cover. The bottom ends of the transmission tubes were located in the receiving space of the main body, and curved such that the longitudinal central line of the bottom ends and the longitudinal central line of the transmission tubes formed a predetermined angle. The outer wall surface of the transmission tubes was secured to the inner wall surface of the holes of the cover by sealing material. The dialysis tube was made of dialysis membrane (Spectrum, 20 mm length, 150 mm outer diameter with a cut-off at a nominal molecular mass of 13 000; Laguna Hills, CA, USA), and immersed in the primary liver cell culture medium. The Petri dish device was housed within a hypoxia chamber. Dialysates were collected and introduced by an on-line injector to a liquid chromatographic system for analysis of pyruvate and lactate, as shown in Fig. 1.

The microdialysis–LC–UV system was comprised of a micropump (CMA-100, CMA, Stockholm,

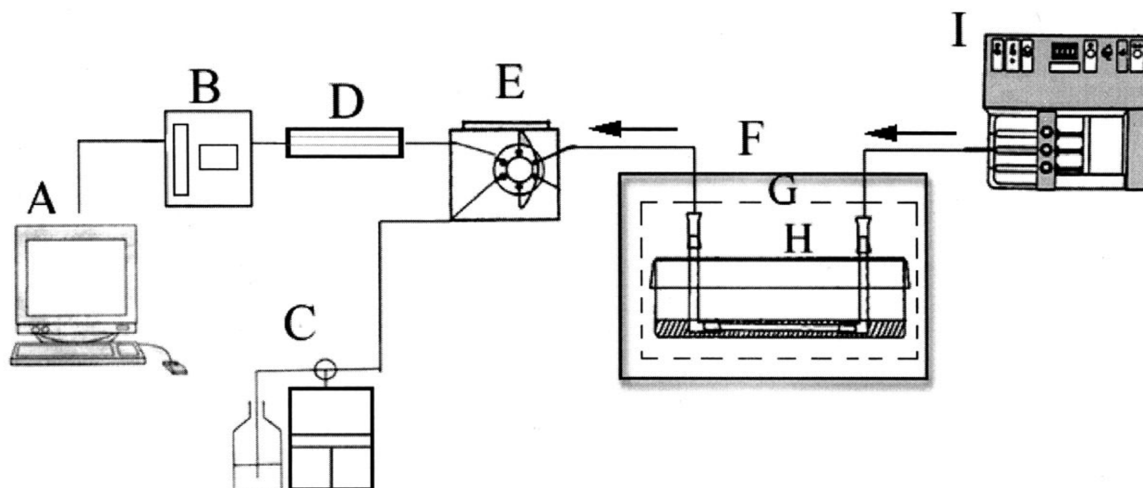

Fig. 1. Schematic diagram of a novel dialysis Petri dish within a hypoxia chamber. An on-line microdialysis–LC–UV system showing: A, the computer data system; B, the HPLC–UV system; C, the HPLC pump; D, column; E, on-line injector; F, incubator; G, hypoxia chamber; H, Petri dish with dialysis membrane; I, syringe pump.

Sweden), an on-line injector (CMA-160), a Microtech LC-pump (Microtech Scientific, Sunnyvale, CA, USA), a BAS UV-116 detector (Bioanalytical Systems, West Lafayette, IN, USA), a Beckman I/O 406 interface (AI-406), and Beckman System Gold Data Analysis Software (Beckman Instruments, Taiwan Branch) for the measurement of pyruvate and lactate. Chromatograms were recorded on the Beckman I/O 406 interface, and analyzed via the Beckman System Gold Data Analysis Software. Dialysis dishes were perfused with culture medium solution at 2 μl/min using a CMA-100 microinfusion pump. Dialysates were collected and injected directly onto the LC–UV system every 30 min by a CMA-160 autoinjector. Following 3-h baseline collection, hypoxia was induced by replacing air with N_2. Primary liver cells under hypoxia were incubated for an additional 3 h. Then, the incubator was deoxygenated for an additional 2 h.

Separation of substances was achieved using a conventional Polypore column (100×4.6 mm I.D.) packed with 10 μm Polypore H (Brownlee Labs, IL, USA). The mobile phase consisted of 4 m*M* sulfonic acid in doubled-distilled water (112 μl concentrated sulfonic acid in 1000 ml distilled water). The mixture was filtered through a 0.22 μm Nylon filter under reduced pressure and sparged by helium for 20 min. The flow-rate was 0.5 ml/min, at a maintained column pressure of ca. 2.1 MPa. The concentrations of pyruvate and lactate in dialysates were calculated by determining each peak area ratio relative to the standard mixture.

The concentration of an analyte in a dialysate is related to the concentration of that analyte in the primary liver cell culture medium. This relationship is called the relative recovery and is usually expressed as a percent value. For in vitro microdialysis recovery, the dialysis device was calibrated in a standard solution containing 100 and 1000 μ*M* of pyruvate and lactate in 4 m*M* sulfonic acid, respectively. The working buffer solution was used as perfusing solution for the microdialysis experiment. The perfusion flow-rate was set at 2.0 μl/min. In vitro recovery values of all analytes at 2.0 μl/min were recorded and validated on each dialysis device prior to experiments. The dead volume (ca. 36 μl) of the microdialysis system from the Petri dish to the autoinjector was also determined. Recoveries of microdialysis devices between 45 and 55% were used to ensure analytical quality. Experimental data were recorded and analyzed via Beckman System Gold Data Analysis Software.

3. Results and discussion

Fig. 2A shows a typical chromatogram of a standard mixture containing pyruvate and lactate. Analysis was completed within 10 min. Fig. 2B–D show typical chromatograms of microdialysates obtained from primary liver cell culture medium at (B) baseline, (C) 1 h after hypoxia began and (D) 30 min after reperfusion, respectively. All components under study were well resolved. Retention times for pyru-

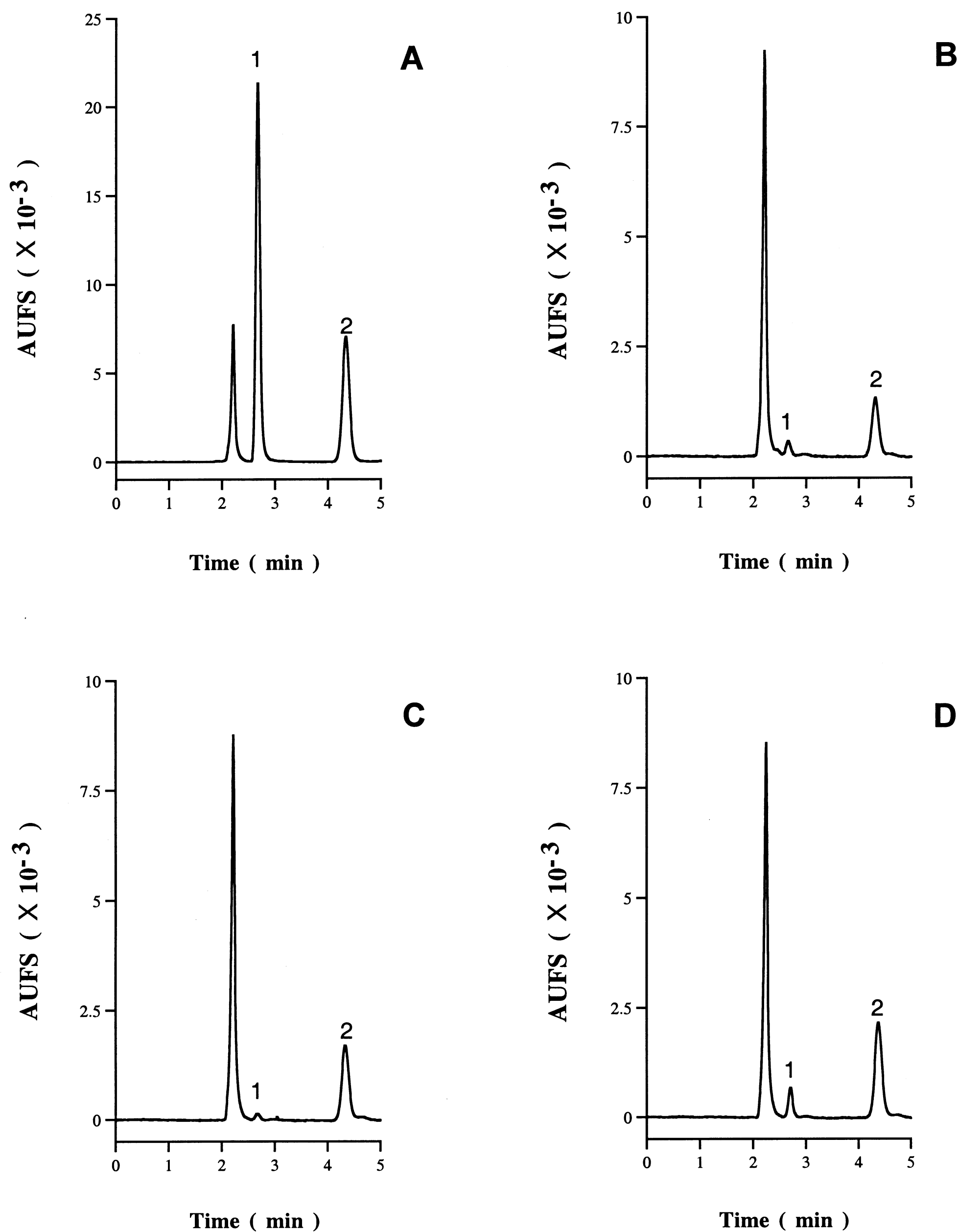

Fig. 2. Typical chromatograms obtained from (A) a standard mixture, (B) a microdialysate at baseline, (C) a microdialysate 1 h after hypoxia began and (D) a microdialysate 30 min after reperfusion (1, pyruvate; 2, lactate).

Table 1
Correlation of peak area with the amount of pyruvate and lactate in standard mixtures (ranging from 1 to 100 μ*M* of pyruvate and from 10 to 1000 μ*M* lactate) measured by the LC–UV system

	Standard curve equation[a]	R^2
Pyruvate	$y = 12\,934x + 0.02$	1.000
Lactate	$y = 766x + 0.01$	1.000

[a] *y*, peak area measured; *x*, amount of analytes in μ*M*.

vate and lactate are identical in Fig. 2A–D. The baseline concentrations of pyruvate and lactate in primary liver cell culture medium were 10.6±5.6 and 607±143 μ*M*, respectively.

Calibration curves were constructed with three standard mixtures (containing ca. 1–100 μ*M* of pyruvate and 10–1000 μ*M* of lactate, $n = 3$) prior to LC–UV analyses on the day of the experiment (Table 1). Concentrations of pyruvate and lactate in dialysates were determined by these calibration curves. The precision of the assays was validated using a standard mixture and pooled dialysates of primary liver cell culture medium (Table 2). The intra-assay variabilities were assessed with 12 replicates at 1-h intervals and expressed as relative standard deviations (RSDs, %). The intra- ($n = 12$) and inter-assay ($n = 6$) variabilities, and the RSDs of all analytes in dialysates, were less than 7.4%. The detection limits (signal-to-noise ratio = 3) of all analytes in the present assay were between 0.2 and 2.0 μ*M*.

Extracellular pyruvate and lactate concentrations in dialysates of primary liver cells did not change much within an 8-h period (data not shown). However, pyruvate concentrations dramatically decreased to 73% during hypoxia, as shown in Fig. 3A. The entire time profiles of pyruvate, lactate, and the lactate/pyruvate ratio obtained from primary liver cell culture medium during hypoxia and reperfusion are shown in Fig. 3A–C. It is evident that these analytes were altered soon after hypoxia. However, the detailed mechanism of hypoxia requires further investigation.

The present method requires no pretreatment of samples. Conventional pretreatment procedures require more than 4 h to prepare eight to 10 samples [13–15]. The cumbersomeness of pretreatment procedures for small volumes of culture medium is still a problem for culture systems. Another advantage of this method is its low detection limits (0.2–0.5 m*M*, typically). This on-line method can increase experimental speed, provide high sensitivity, minimize required sample volume, enhance detection limits, and decrease degradation of the analyzed compounds.

In conclusion, the dialysis device used in this study is capable of examining extracellular lactate and pyruvate with minimal disruption of the normal growth of cells in primary liver cell culture medium. The device also avoids contamination of the cell culture and minimizes the disturbance of the closed dynamic system, particularly within a hypoxia chamber, over a prolonged period of time. Microdialysis samples are relatively free of macromolecules and can be analyzed directly via LC. As a result, the novel dialysis device is relatively simple in construction and is compatible with an on-line injection analyzer. The present system is relatively efficient, cost-effective, and less vulnerable to human error, as compared with conventional systems in which a number of Petri dishes are used. Indeed, the

Table 2
Analytical precision on the stabilities of intra-assay ($n = 12$, at 1-h intervals) and inter-assay ($n = 6$, on 6 consecutive working days) variabilities of standard mixtures and primary liver cell dialysates in the LC–UV system

	Pyruvate		Lactate	
	Conc. (μ*M*)	RSD (%)	Conc. (μ*M*)	RSD (%)
Intra-assay				
Standard mixture in 4 m*M* H_2SO_4	10.0±0.1	1.3	100.4±2.1	2.0
Primary liver cells pooled dialysates	2.7±0.2	7.4	834±10	1.2
Inter-assay				
Standard mixture in 4 m*M* H_2SO_4	10.0± 0.2	1.7	100.0±3.3	3.3

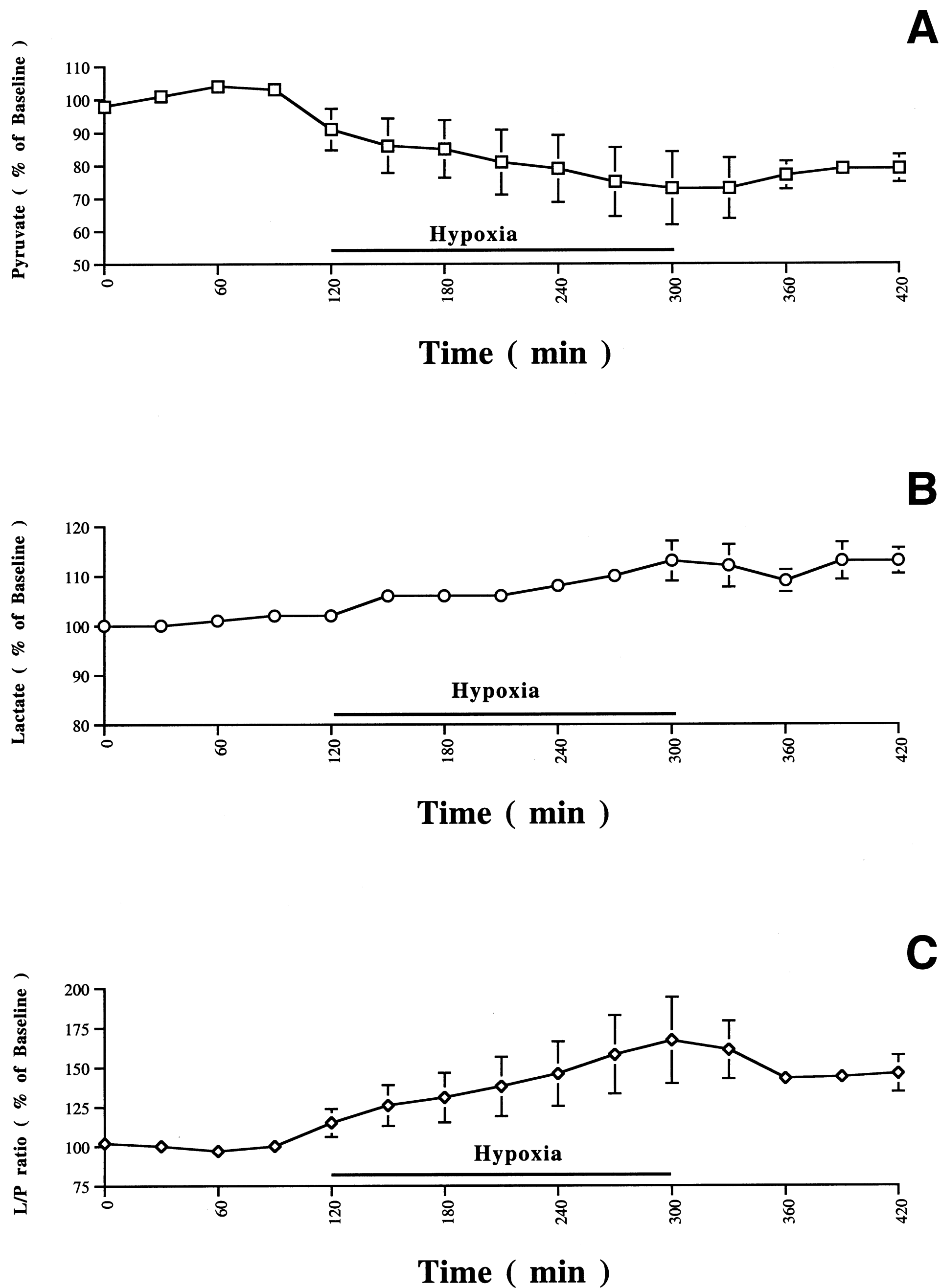

Fig. 3. The entire time profiles of (A) pyruvate, (B) lactate and (C) the lactate/pyruvate ratio obtained from primary liver cell culture medium by the novel microdialysis sampling system.

present system minimizes pretreatment procedures for sample preparation, reduces possible contamination from sampling of the culture medium, and enhances the detection sensitivity of lactate and pyruvate in primary liver cell culture medium. Furthermore, this novel microdialysis device can be

applied to the measurement of chemical substances in other culture cell systems.

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