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Rapid on-line microdialysis hyphenated technique for the dynamic monitoring of extracellular pyruvate, lactic acid and ascorbic acid during cerebral ischemia

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Abstract

Rapid on-line microdialysis coupled with liquid chromatography was developed for the continuous monitoring of brain neurochemicals during cerebral ischemia. Isocratic separation of these analytes was achieved within 3 min, hence, over 80 analyses could be performed in a 4-h experiment. The dead volume of the microdialysis system was estimated to be less than 10 μl. The detection limits of the present assay, at a signal-to-noise ratio of five, were 2.0, 0.2 and 0.5 μ*M*, for lactic acid, pyruvate and ascorbic acid, respectively. To validate this assay, a transient ischemia was produced by occlusion of two common carotid arteries for 10 min in an anesthetized gerbil. A microdialysis probe was inserted into the striatum of the gerbil to simultaneously monitor pyruvate, lactic acid and ascorbic acid during cerebral ischemia. Significant and dynamic changes in these analytes could be resolved in 3-min intervals. This rapid assay can be used as a tool to study dynamic changes in neurochemicals of the brain, such as during cerebral ischemia.

Keywords: Pyruvate; Lactic acid; Ascorbic acid

1. Introduction

Microdialysis was introduced two decades ago for sampling neurochemical substances from the extracellular fluid of the brain [1–3]. Today, it is one of the most widely used techniques for in vivo sampling of the microenvironment of the brain. On-line microdialysis provides for automatic sampling and the acquisition of dynamic information on the brain, such as how events or processes are reflected in the local compartments [4,5].

Transient or prolonged cerebral ischemia results in low oxygen and glucose supply and causes decreased adenosine triphosphate (ATP) formation [6,7]. Various ATP-driven membrane-bound pumps or reuptake processes that usually work in the homeostasis of important metabolites or ions become retarded during cerebral ischemia. Moderate-to-severe neuronal damage might occur following these events. In addition, secondary neuronal injury might occur, becoming worse during reperfusion or after reperfusion, when the above pumps or reuptake channels resume. The selective vulnerability of certain neurons to cerebral ischemia is well established morphologically [8–10]. Previous studies of biochemical processes have mainly been based on whole brain

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homogenates from experimental animals [11–13]. Findings obtained by such methods may not be directly applicable to some areas of brain research. By probing the extracellular compartments, the question has arisen as to whether and to what extent neurochemicals are formed, consumed locally or derived from other sources, such as glial cells, neuronal tissues and the cerebral circulation [14].

Lactic acid levels, pyruvate levels and the lactic acid–pyruvate ratio of the brain have been advocated for estimation of the severity of stroke and also as tools for prognostication of the outcome [15,16]. Lactic acid and ascorbic acid are produced by nerve tissues, including nervous and glial cells under cerebral ischemic conditions [14–16]. Cerebral ischemia might damage the integrity of the cellular membrane of brain tissues and thus,, intracellular lactic acid and ascorbic acid are released into the extracellular space [14]. Ascorbic acid release may also be regulated by glutamate transporters, which are altered during cerebral ischemia. Under anaerobic conditions, pyruvate is reduced to lactic acid in a reverse reaction that is catalyzed by lactic acid dehydrogenase. Changes in lactic acid, pyruvate, ascorbic acid and in the lactic acid–pyruvate ratio may serve as biochemical markers of impending cerebral ischemia.

Significant ischemic damage has been demonstrated with transient ischemic insults as brief as 5 min. It seems reasonable to expect that ischemic mediators may begin to occur with a brief transient ischemia. Previous studies have not concentrated on the nature of the earliest ischemia and reperfusion-related changes in lactic acid, pyruvate, ascorbic acid and in the lactic acid–pyruvate ratio. Understanding these initial changes may make it easier to explore the primary cellular events that eventually lead to ischemic injury or delayed neuronal death. The simultaneous determination of pyruvate, lactic acid and ascorbic acid is important for the investigation of the brain injury mechanism in the earliest stages of ischemia and reperfusion [14–16].

Conventional microdialysate sample collection has varied from 15 to 30 min in cerebral ischemia in previous experiments [14–17]. However, after induction of cerebral ischemia, changes in brain extracellular ion concentrations show two major phases. In the first phase, lasting 1.5 to 2 min, extracellular potassium increases slowly and calcium and sodium concentrations decrease [18]. The major breakdown in ion homeostasis (the beginning of the second phase) occurs when phosphocreatine and ATP concentrations approach zero. There has been difficulty in applying conventional microdialysis assays to the investigation of extracellular conditions and detailed mechanisms over such short time intervals. Thus, there is a need to develop a rapid assay (less than 3 min) to resolve these short time-interval events.

The aim of the present study was to investigate a rapid change in an ischemic event via a rapid on-line microdialysis hyphenated technique for the simultaneous determination of pyruvate, lactic acid and ascorbic acid. The basic idea behind this study was that monitoring substances in the extracellular fluid using microdialysis could provide useful information on energy metabolic disturbance with high temporal and spatial resolution.

2. Experimental

Standard stock solutions of pyruvate and lactic acid 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. Ascorbic acid was prepared fresh daily at a concentration of 100 μ*M*. In vitro recovery was also performed in a standard mixture containing pyruvate, lactic acid and ascorbic acid, to determine the recoveries of all analytes and the dead volume of the microdialysis system, as shown in Fig. 1.

The gerbil was anesthetized with chlorohydrate (400 mg/kg), administered intraperitoneally, and its body temperature was maintained at 37°C with a heating pad (CMA/150). Supplements of chlorohydrate (100 mg/kg) were given as needed. Both common carotid arteries, exposed through a ventral midline incision in the neck, were carefully separated from the vago-sympathetic trunks and loosely encircled with sutures. O-rings were sutured in carotid arteries for later occlusion. The gerbil's head was mounted on a stereotaxic apparatus (Davis Kopf Instruments) with the nose bar positioned 4.0 mm below the horizontal line. Following a midline

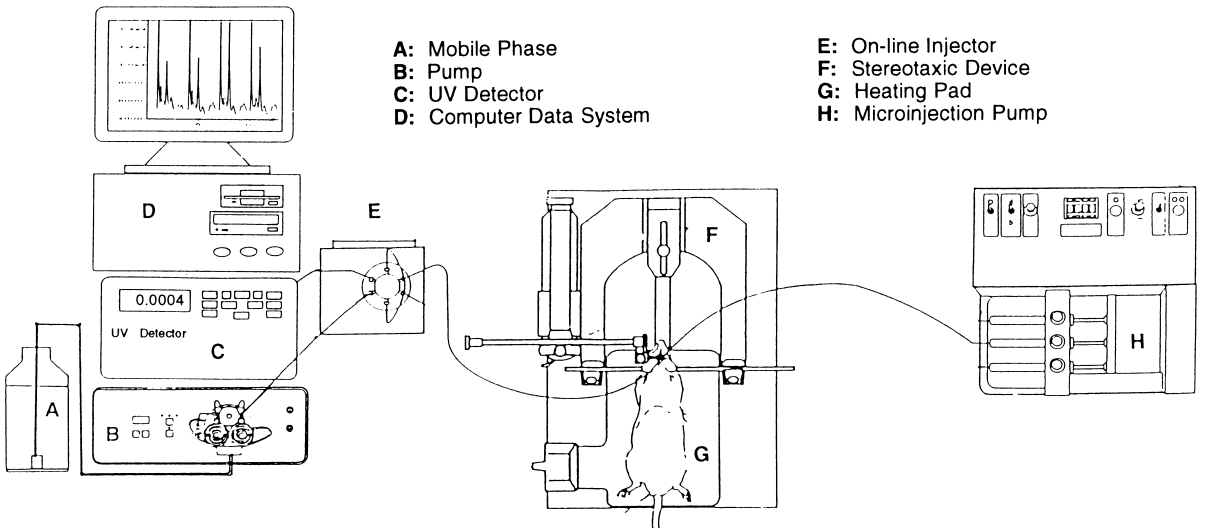

Fig. 1. Schematic diagram of an on-line microdialysis system showing (A) the mobile phase, (B) the pump, (C) the UV detector, (D) the computer data system, (E) the on-line injector, (F) the stereotaxic frame, (G) the heating pad and (H) the microinjection pump.

incision, the skull was exposed and one burr hole was made on the skull for the insertion of a dialysis probe (4 mm in length, CMA/12, Carnegie Medicin, Stockholm, Sweden). A microdialysis probe was stereotaxically implanted into the striatum (AP 0.5 mm, ML −2.5 mm, DV −6.0 mm from bregma). Both carotid arteries were pulled by 6 g weights to occlude the circulation for 10 min, producing a transient cerebral ischemia. Then, reperfusion was performed for an additional 2 h [19,20].

Dialysis probes were perfused with Ringer's solution (147 m*M* Na^+; 2.2 m*M* Ca^{2+}; 4 m*M* K^+, pH 7.0) at 2 μl/min using a CMA/100 microinfusion pump. Dialysates were collected every 3 min in a 5-μl loop in a CMA/160 autoinjector. Dialysates (5 μl) were directly injected onto an LC system with a UV detector (BAS UV-116A, Bioanalytical Systems, West Lafeyette, IN, USA) for the determination of lactic acid, pyruvate and ascorbic acid. Separation of these substances was achieved using a conventional Polypore column (100×4.6 mm I.D.) packed with 10 μm Polypore H (Brownlee Lab, IL, USA).

The mobile phase consisted of 4 m*M* sulfonic acid in doubled distilled water (112 μl of concentrated sulfonic acid in 1000 ml of distilled water). The mixture was filtered through a 0.22-μm Nylon filter under reduced pressure and sparged with helium for 20 min. The flow-rate was 500 μl/min, at a maintained column pressure of ca. 2.1 MPa. The concentrations of pyruvate, ascorbic acid and lactic acid in dialysates were calculated by determining each peak area ratio relative to the standard mixture. The identity of these peaks on the chromatogram was confirmed by their retention times and a superimposed technique that was provided by HP (Hewlett-Packard 3365 Series II ChemStation, Taiwan Branch, Taipei, Taiwan).

3. Results and discussion

Fig. 2A shows a typical chromatogram of in vitro recovery of a microdialysis probe in a standard mixture containing 0.5, 0.5 and 5.0 m*M* pyruvate, ascorbic acid and lactic acid. Analysis was completed within 2 min. In order to demonstrate the differences between each analysis, five consecutive analyses were recorded on one chromatogram by HP Chemstation computer software. The third to the fifth intervals were almost identical, as shown in Fig. 2A. Stable recoveries of microdialysis probes were obtained in the third interval and thereafter. The dead volume of this microdialysis system was estimated to be less than 10 μl. Therefore, the representative

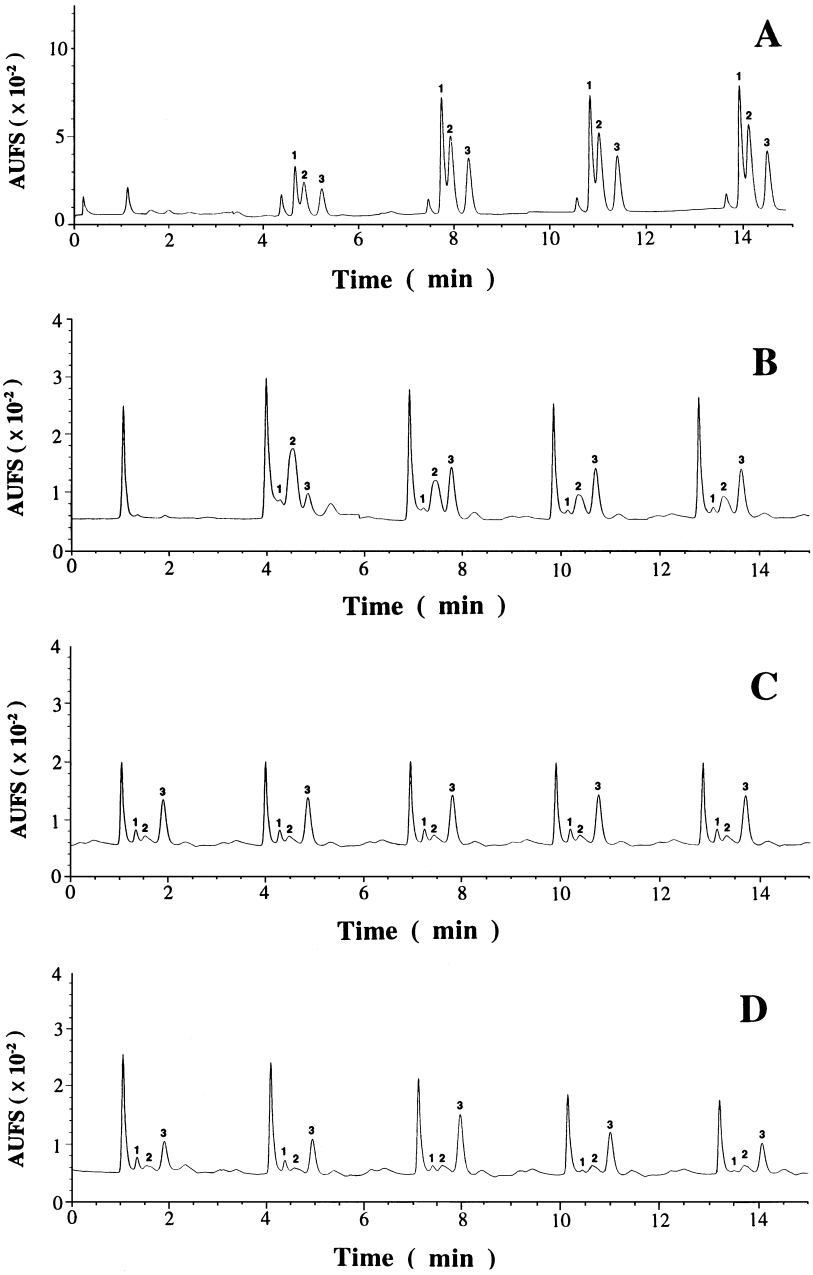

Fig. 2. Typical chromatograms obtained from (A) in vitro recovery of a standard mixture containing (1) pyruvate, (2) ascorbic acid and (3) lactic acid; (B) implantation of a microdialysis probe into the right striatum; (C) basal levels prior to a transient ligation and (D) 10-min ligation and reperfusion in a gerbil.

analysis of an event was delayed by approximately 6 min (or two intervals).

Calibration curves were obtained with six standard mixtures each of pyruvate (2, 20, 50, 100, 200 and 500 μM), ascorbic acid (1, 10, 25, 50, 100 and 250 μM) and lactic acid (20, 200, 500, 1000, 2000 and

Table 1
Correlations of known amounts of standard mixtures (range: 0.1–5 m*M*) with chromatographic peak areas measured by a liquid chromatography (LC)–UV system (wavelength set at 214 nm)

	Standard curve equation[a]	R^2
Pyruvate	$Y=1695.6X+3.1$[b]	1.000
Lactic acid	$Y=105.9X+7.0$	1.000
Ascorbic acid	$Y=2701.6X-5.1$	1.000

[a] Y=Peak-area measurement; X=amount of analytes, in μ*M*.
[b] Analyte amounts in the range of 2–500 μ*M* (pyruvate), 20–5000 μ*M* (lactic acid) and 1–250 μ*M* (ascorbic acid).

5000 μ*M*), prior to LC analysis of striatal microdialysates. The amounts of each injected analyte were linearly related to the chromatographic area obtained from standard mixtures over a large range of concentrations. The correlations (R^2) for pyruvate, ascorbic acid and lactic acid, on typical calibration curves, were linear ($R^2=1.000$, as shown in Table 1).

The precision and stability of the assays were tested using standard mixtures (containing 0.1 m*M* pyruvate, 0.1 m*M* ascorbic acid and 1.0 m*M* lactic acid) in various matrices (0.1 *M* HCl, 4.0 m*M* sulfonic acid and Ringer's solution) and a pooled microdialysate (Table 2). The intra-assay variabilities were assessed with 12 replicates at 1-h intervals and expressed as coefficients of variation (C.V., %). In general, the C.V.s for pyruvate, ascorbic acid and lactic acid in various matrices and the pooled dialysate were acceptable (<3.0%). The inter-assay variabilities were assessed with a standard mixture containing 0.1 m*M* pyruvate, 0.1 m*M* ascorbic acid and 1.0 m*M* lactic acid over five consecutive working days. The C.V.s for pyruvate and lactic acid were much lower (2.38 and 2.50%) than for ascorbic acid (6.05%) in the chromatographic measurements. Ascorbic acid had to be prepared daily by weighing a small amount of the drug, leading to a higher C.V. value, whereas pyruvate and lactic acid could be prepared from a stock solution. The detection limits (signal-to-noise ratio= 5) of all analytes in the present assay were between 0.2 and 2 μ*M* (0.2 μ*M* for pyruvate, 0.5 μ*M* for ascorbic acid and 2.0 μ*M* for lactic acid).

Fig. 2B shows a typical chromatogram of five consecutive microdialysates from a gerbil striatum after implantation of a microdialysis probe. The retention times of each peak corresponding to pyruvate, ascorbic acid and lactic acid in Fig. 2B were identical to those in Fig. 2A. All of the analytes studied were well resolved. Some interfering peaks (at ca. 2.3 and 3.3 min) are shown in Fig. 2B. Three-minute intervals were chosen to avoid interference from the peak at 2.3 min. The second interfering peak at 3.3 min was eluted right before the non-elution peak of the second run, therefore, it did not affect the chromatographic separation of any of the analytes.

After implantation of a microdialysis probe, ascorbic acid increased rapidly, to 700% of the basal concentration, and was maintained at relatively stable levels within 15 min of perfusion (Fig. 2B). The second interval, within the delayed dead volume, contains a higher level of ascorbic acid (160%) than the third interval on the chromatogram. These data indicate that ascorbic acid was quickly released right after implantation of the microdialysis probe. Thereafter, ascorbic acid decreased gradually to a basal concentration of 33 μ*M* within 90 min of perfusion. Pyruvate increased in the first 20 min and was

Table 2
Analytical precision of various standard mixtures and a pooled dialysate on the intra- ($n=6$) and inter-assay ($n=5$, over five consecutive working days) of the LC–UV system

Matrices	Pyruvate	Lactic acid	Ascorbic acid
Intra-assay			
0.004 *M* H_2SO_4	2.02	0.63	0.72
0.01 *M* HCl	2.05	0.89	0.34
Ringer's solution	2.04	0.44	1.27
Pooled dialysate	1.79	0.33	1.05
Inter-assay			
Ringer's	2.38	2.50	6.05

maintained for 30 min, whereas lactic acid approached stable levels within 10 min of perfusion. The measurements of pyruvate, ascorbic acid and lactic acid in such small volumes and with such low detection limits are of great analytical potential in microdialysis experiments. In the anesthetized gerbil, 2 h after implantation of the probe, basal concentrations of all analytes in striatal dialysates were obtained. The basal concentrations of pyruvate, ascorbic acid and lactic acid were 4.0, 33 and 78.2 μ*M*, respectively (Fig. 2C). Ligation of two common carotid arteries producing a transient cerebral ischemia was performed after obtaining basal levels of all analytes.

Lactic acid increased to 184% of the basal level in the first 3 min interval and then decreased to 82% of the basal level after 15 min following a 10-min transient cerebral ischemia (Fig. 2D). The mechanism by which ischemia causes increased lactic acid levels in the extracellular space probably reflects both an increased release of lactic acid into the extracellular space as well as a decreased removal of lactic acid from the extracellular space. Removal of lactic acid from the extracellular space is largely due to an uptake mechanism, which is an ATP-dependent process. Ischemia is known to cause ATP depletion within 5 min. Therefore, the lactic acid uptake mechanism is blocked and lactic acid levels accumulate. In fact, the potassium levels in the extracellular space are known to increase rapidly, reaching maximal levels of up to 60 m*M* within 3–4 min of cerebral ischemia.

The entire time profiles obtained for extracellular pyruvate, lactic acid, ascorbic acid and the lactic acid–pyruvate ratio in the striatum before and after a 10-min transient cerebral ischemia are shown in Fig. 3. It is evident that these analytes varied very soon after transient ischemia and reperfusion. After 10 min of reperfusion, lactic acid (Fig. 3B) and ascorbic acid (Fig. 3D) gradually increased to a plateau level, whereas pyruvate reached its basal levels in 120 min. The time-frame overlaps showed a lag in pyruvate in the present study (Fig. 3A). The consumption of pyruvate might be delayed for a short period of time (3–4 min) because of the blockade of some transport. Soon after this event, pyruvate decreased drastically to 16% of the basal level after 15 min, and then gradually rose to 90% of the basal level within 120 min (Fig. 3A). The basal lactic acid–pyruvate ratio was about 30 prior to the ligation (Fig. 3C). The ratio increased significantly, to 140, at the first 20 min interval after ligation. The ratio gradually decreased to about 40 within 120 min. In general, increased lactic acid and decreased pyruvate production by tissues were demonstrated during cerebral ischemia, because of decreased regional cerebral blood flow. However, our data support a possible biphasic increase in the lactic acid–pyruvate ratio. The early phase of increasing lactic acid–pyruvate ratio could be stimulated by a decreased cerebral flow, whereas the second phase of increasing lactic acid–pyruvate ratio could be generated solely by a secondary injury caused by reperfusion. Ascorbic acid demonstrated a two-phase pattern of increase in early ischemia and later reperfusion periods (Fig. 3D). A 30% increase in the first phase was clearly demonstrated as an increase in ascorbic acid in the microenvironment during the stress of transient cerebral ischemia. The decrease in ascorbic acid in the first phase was predominantly due to the reperfusion of blood flow causing a dilution of local ascorbic acid levels. Thereafter, the ascorbic acid level increased drastically, up to 260% of basal level at the peak of 60 min. Ascorbic acid levels decreased slowly to 160% of the basal level within 120 min. These data are in agreement with those of other investigators [20]. These biphasic phenomena can be seen in such short intervals only by the present rapid on-line microdialysis sampling techniques. However, the detailed mechanism of the biphasic increases in ascorbic acid and in the lactic acid–pyruvate ratio in transient cerebral ischemia requires further investigation.

The gerbil brain lacks the connection between the carotid and vertebrobasilar circulation, which makes the circle of Willis incomplete [21]. Little anteroposterior blood flow exists in the gerbil brain. Therefore, simple carotid artery ligation will prevent blood flow to the anterior portion of the brain. This cerebrovascular plan has made the gerbil brain an excellent model for studying transient cerebral ischemia. These chromatograms and data clearly demonstrate gerbil striatal extracellular energy-related metabolites and an antioxidant, ascorbic acid, in a near ‘real time’ manner. The studies yielded the entire time profiles of dynamic information on chemical changes in an experimental animal subjected to a brief transient cerebral ischemia.

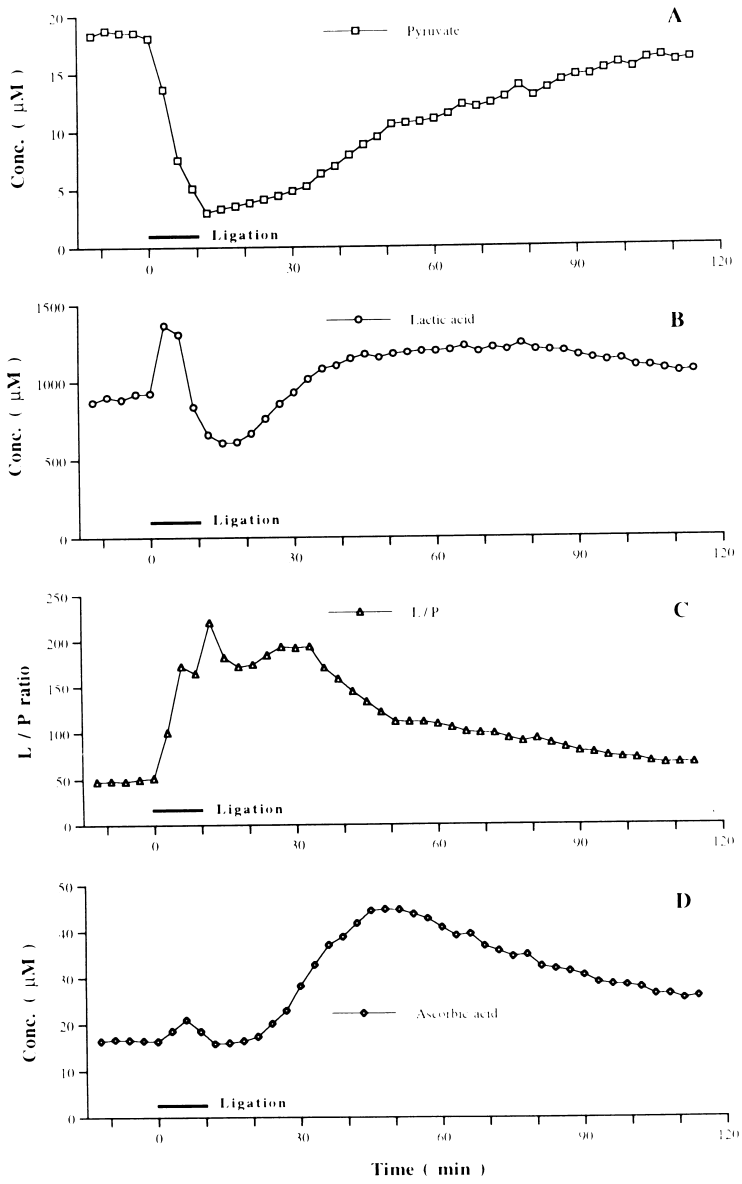

Fig. 3. Entire time profiles obtained for extracellular (A) pyruvate, (B) lactic acid, (C) lactic acid–pyruvate ratio and (D) ascorbic acid in gerbil striatum before and after a 10-min transient cerebral ischemia.

4. Conclusion

Rapid on-line microdialysis coupled with liquid chromatography was developed for the continuous monitoring of striatal lactic acid, pyruvate and ascorbic acid levels within 3 min. The detection limits of the present assay, at a signal-to-noise ratio of five, were 2.0, 0.2 and 0.5 μ*M*, for lactic acid, pyruvate and ascorbic acid, respectively. Significant and dynamic changes in these analytes were observed in an anesthetized gerbil subjected to a probe insertion and a 10-min transient ischemia. Biphasic increases in ascorbic acid and the lactic acid–pyruvate ratio in transient cerebral ischemia can be clearly demonstrated by the present rapid on-line microdialysis sampling techniques. The use of a

rapid on-line microdialysis hyphenated technique provides a near 'real-time' monitoring of chemical changes with a great potential for investigating detailed intracranial dynamics. An assay by which the energy states of the brain can be monitored is valuable in a number of acute experimental animal models and clinical situations. On-line bedside chemical analysis devices can be made available, particularly for patients in intensive care units or emergency rooms. Furthermore, recent research has pointed out several cerebral ischemic processes leading to delayed neuronal cell death, such as lactic acidosis. A number of anti-ischemic drugs are currently being evaluated in cerebral ischemic animal models and clinical trials. Thus, the present assay could be applied in these fields to evaluate lactic acidosis in a near 'real-time' manner.

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