

Determination of Lactate with Liquid Chromatography/Electrochemistry Coupled with a Lactate Oxidase IMER

Liu Yang*,
Gary Overdorf, and
Pete Kissinger
Bioanalytical Systems
West Lafayette, IN
47906-1382
Phone: (765) 463-4527
Fax: (765) 497-1102
E-mail:
pete@bioanalytical.com

* Present Address:
Eli Lilly
Tippecanoe Labs
Lafayette, IN

An LCEC (liquid chromatography/electrochemistry) method was developed for the determination of lactate. The method was based on the combination of anion exchange separation, lactate oxidase IMER (immobilized enzyme reactor) oxidation of lactate to H₂O₂, and electrochemical detection of H₂O₂ with a peroxidase modified electrode. This method can separate lactate very well from oxidizable anions such as ascorbate. The detection limit of the method is 10 μM of lactate with 5 μL injection, and the linear range is 10 μM to 1.5 mM. Lactate in rat subcutaneous microdialysate was determined with this method.

There have been several methods published for the determination of lactate using liquid chromatography. One method (1) involves reverse-phase separation with a phosphate buffer at pH 3 as the mobile phase. Following the separation, the lactate is converted to hydrogen peroxide by a lactate oxidase IMER and then detected amperometrically. The optimal pH for the enzyme lactate oxidase is around 7, and at pH 3 the enzyme is basically not functioning. Therefore, for this system, a second pump and an on-line mixer have to be used to increase the pH of the mobile phase after the separation column to keep the enzyme in the IMER active. This makes the system rather complicated. Another method (2) also involving reverse-phase separation with low pH phosphate buffer uses UV detection instead of the electrochemical detection coupled with a lactate oxidase IMER. Lactate does not absorb in the usual UV-VIS region, and the direct detection of

lactate has to be conducted in the low wavelength UV region. This makes serious background interferences likely. There is also a reverse-phase separation using a mixture of phosphate and acetonitrile as mobile phase. For this separation, there are two detection methods: UV-VIS spectrophotometry (3) and fluorimetry (4). For both detection methods, the lactate has to be derivatized and therefore the methods are not convenient. Ion chromatography has also been used for the separation of lactate. The two published methods use conductimetric (5) and acoustic wave (6) detection. The conductimetric method requires several ion-exchange steps to reduce the background, resulting in a very complicated system. Besides, the chromatogram usually involves many anionic peaks that make data analysis difficult. The acoustic wave method, on the other hand, is limited by its lack of commercial availability and difficult data interpretation. In this paper we

describe the development of a simple and practical LCEC method for the determination of lactate.

Experimental

Materials

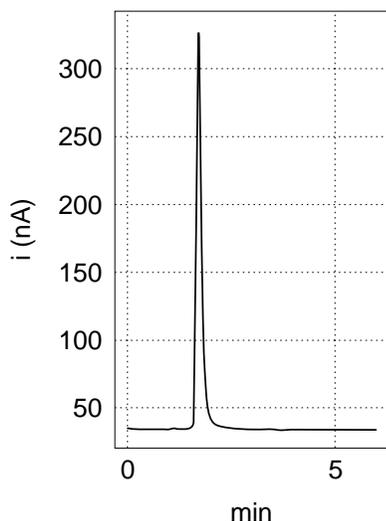
Lactate oxidase was purchased from Genzyme (Cambridge, MA). L-(+)-lactic acid, sodium salt was purchased from Calbiochem Corporation. All other chemicals were analytical reagent grade from Sigma or Aldrich. Artificial CSF solution was prepared as follows: 126 mM NaCl, 27.5 mM NaHCO₃, 2.4 mM KCl, 0.5 mM KH₂PO₄, 1.1 mM CaCl₂, 0.85 mM MgCl₂, 0.5 mM Na₂SO₄, pH 7.5. All standard lactate solutions were prepared with the artificial CSF solution.

Microdialysis

The rat was anesthetized with an intraperitoneal injection of 1 mL/kg of KX (10 mL ketamine (100 mg/mL) + 1 mL xylazine (100 mg/mL)). The microdialysis probe

F1

Chromatogram of a 5 μL injection of a 0.1 mM lactate standard with a 10 cm anion-exchange column and 50 mM Na_2HPO_4 , pH 8.0 as the mobile phase. Flow rate = 1 mL/min. Room temperature.



(DL-5, MF-7051, BAS) was placed in an introducer cannula (MF-7021, BAS). The animal's fur was clipped from the insertion site on the back at the base of the neck. Small incisions were made at both sites. The cannula was inserted at the neck incision and advanced under the skin for about 7 cm. The cannula was then pulled back out, leaving the fiber portion of the probe under the skin. The probe was sutured in place and perfused with artificial CSF solution at 5 $\mu\text{L}/\text{min}$ with a BAS Bee syringe drive (MD-1001). During microdialysis sampling, the awake unrestrained rat was housed

in the "BAS Beekeeper" containment and swivel system (MD-1575, BAS). All of the experimental protocols were approved by the BAS Animal Care and Use Committee.

Chromatography

The LCEC determination of lactate was performed with a BAS 480 liquid chromatography system and a peroxidase enzyme electrode (MF-2095, BAS) poised at +100 mV vs. Ag/AgCl. An experimental polymeric anion exchange column (10 \times 0.2 cm, 10 μm) packed at BAS was used for the separation. The mobile phase was either 50 mM Na_2HPO_4 , pH 8.0 or 50 mM LiAc, pH 8.25. A lactate oxidase IMER made by BAS was used between the separation column and the peroxidase electrode detector for the oxidation of lactate to hydrogen peroxide. In the reverse-phase separation test, a BAS Phase II ODS-3 column (MF-6215) was used. The mobile phase was 20 mM NaH_2PO_4 and 0.05% (v/v) dimethylhexylamine, pH 5.5 at a flow rate of 0.5 mL/min. All other conditions were the same as with the ion-exchange separation.

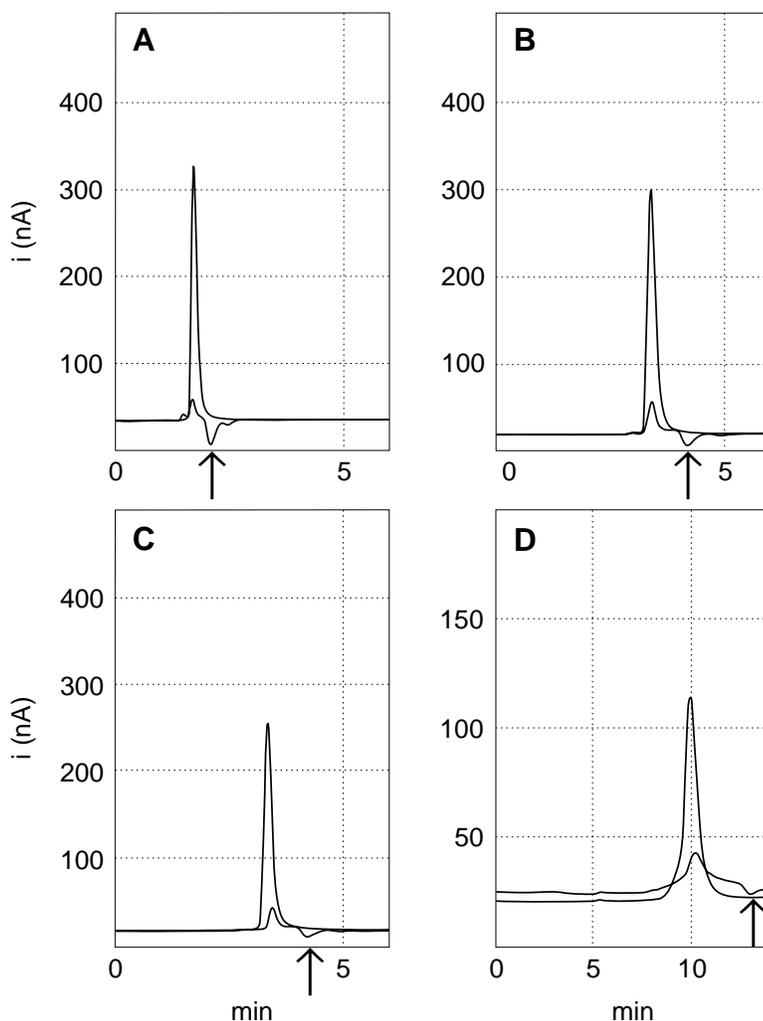
Unless stated otherwise, all experiments were carried out at room temperature.

Results and Discussion

Because there were two enzymes (lactate oxidase in the IMER and horseradish peroxidase at the electrode) involved in our LCEC system for lactate determination, a phosphate buffer at pH 8, which is generally suitable for enzymes, was first used as the mobile phase. **F1** shows a typical chromatogram of a standard lactate/CSF solution when the phosphate buffer was used with the ion-exchange column, the lactate oxidase IMER, and the peroxidase electrode. The graph is clean and simple, however, the separation efficiency of the system was poor. The retention of the lactate was not long enough. With this system, the lactate could not be separated from ascorbate as shown in **F2A**, where

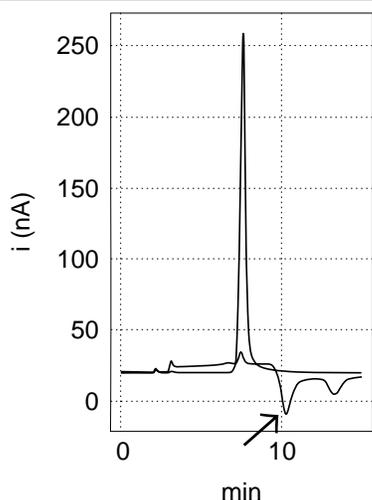
F2

Overlapping chromatograms of 0.1 mM, 5 μL lactate (upper) and ascorbate (lower) A) At the same condition as in F1; B) At the same condition as in F1 except that the flow rate was 0.5 mL/min; C) At the same condition as in B except that the temperature was 35°C; D) With 20 cm anion-exchange column and at a flow rate of 0.4 mL/min. The arrow indicates the negative ascorbate peak

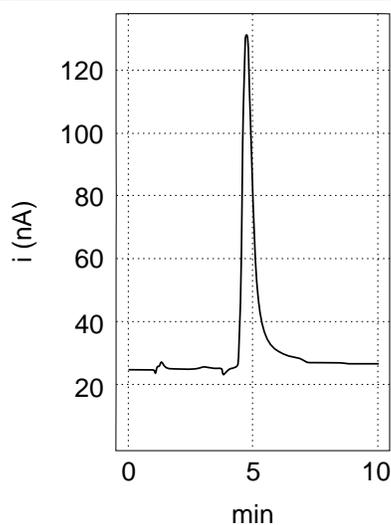


F3

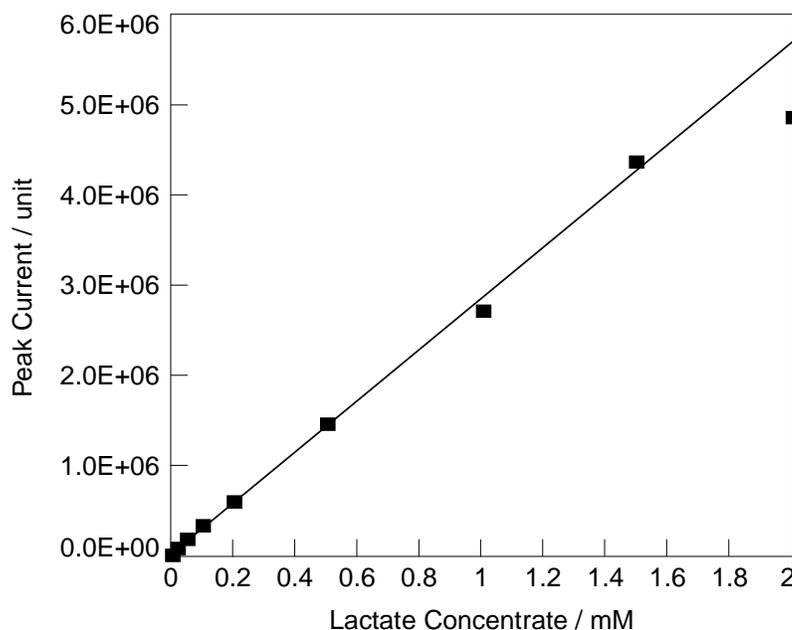
Chromatogram of standard 0.1 mM lactate and ascorbate solutions, 5 μ L, with a 10 cm anion-exchange column and 50 mM LiAc, pH 8.3 as the mobile phase. Flow rate = 0.5 mL/min. Room temperature.

**F4**

Chromatogram of a standard lactate solution of 0.1 mM, 5 μ L, with an ODS-3 column and 20 mM NaH_2PO_4 , 0.05% (v/v) dimethylhexylamine, pH 5.5 as the mobile phase. Flow rate = 0.5 mL/min. Room temperature.

**F5**

Peak current as a function of standard lactate solutions. 5 μ L sample injected; 10 cm anion-exchange column; 50 mM LiAc, pH 8.3 mobile phase. Flow rate = 0.5 mL/min. Room temperature.



the chromatograms of a lactate solution and an ascorbate solution taken separately were overlapped. In this graph, the large positive peak is from the 0.1 mM lactate solution; the negative peak is from the 0.1 mM ascorbate solution. The ascorbate peak is actually on the shoulder of the lactate peak. The small positive peak from the ascorbate solution is from the blank solution and will be discussed below.

For ion-exchange separation, there are several strategies for improving the separation efficiency, including a lower flow rate, a higher temperature, and a longer column. Therefore, first, the flow rate was reduced from 1 mL/min to 0.5 mL/min with all other conditions being the same as for **F2A**. As shown in **F2B**, a lower flow rate increased the retention time and improved the separation of lactate and ascorbate. Then the temperature was increased to 35°C by using a temperature controller and a heater for the columns. Since the enzyme in the IMER could not tolerate high temperatures, the temperature increase cannot be very dramatic. As shown in **F2C**, the increase in temperature did not make any significant difference in the separation ef-

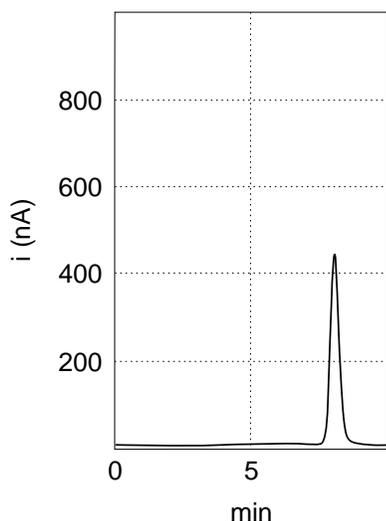
iciency. Finally, two separation columns were used instead of one to increase the column length. Due to the increased back pressure with the longer column, a flow rate of 0.4 mL/min was used in this test. The retention for lactate was much longer with the longer column. However, the peak was very broad and not well shaped. Overall, with the phosphate buffer as the mobile phase, the 10 cm ion-exchange column with a flow rate at 0.5 mL/min and at room temperature worked best.

For further improvement of the separation, a 50 mM LiAc solution of pH 8.3 was tested as the mobile phase. The reason for this change of mobile phase is that Li^+ is a much weaker cation than Na^+ and thus has less interaction with lactate, and acetate is weaker than phosphate and therefore has less competition with lactate. Both of them may contribute to stronger retention of the lactate on the column. **F3** shows the chromatograms of lactate and ascorbate obtained at the same condition of **F2B** except that the sodium phosphate buffer was replaced with the lithium acetate buffer as the mobile phase. As can be seen in **F3**, with LiAc buffer, the retention of lactate is much longer while the peak is still in very good shape, and the lactate and ascorbate are very well separated.

Besides ion-exchange separation, a reverse-phase column with an ion-pairing agent in the mobile phase was also tested for the lactate determination. Reverse-phase separations usually have a higher separation efficiency than ion-exchange separations, and by using an ion-pairing agent, low pH can be avoided for the separation. A BAS ODS-3 column with a phosphate buffer with dimethylhexylamine as mobile phase was tested. As shown in **F4**, however, the lactate peak has very serious tailing under these conditions. The result from the reverse-phase separation system is simply not as good as that from the

F6

Chromatogram of 5 μL rat subcutaneous microdialysate, with 10 cm anion-exchange column and 50 mM LiAc, pH 8.3 as the mobile phase. Flow rate = 0.5 mL/min. Room temperature.



ion-exchange separation system, although it can be used.

Based on the above results, the use of an ion-exchange column with LiAc buffer as mobile phase, a lactate oxidase IMER, and a peroxidase electrode offered the most satisfactory determination of lac-

tate. One small problem with this method for the lactate assay was that with the injection of almost any blank solution, there was always a small peak occurring at exactly the same position as the lactate. It is not clear whether this peak is due to residual lactate in the injector or syringe. This phenomenon recurred even with thorough cleaning of the injector and syringe. This peak from blank solutions limited the detection limit for lactate to 10 μM . Considering the relatively high lactate concentration in most biological samples, however, this method is still practical. The upper limit of the dynamic range of the detection is about 1.5 mM, as shown in **F5**. **F6** shows a typical chromatogram when this method was used to determine lactate in rat subcutaneous microdialysate. The lactate determined in the microdialysis samples varied from 0.8 to 1.4 mM.

Conclusion

The combination of an anion-exchange column with LiAc buffer as mobile phase, a lactate oxidase IMER, and a peroxidase electrode is a practical configuration for the determination of lactate in biological samples.

References

1. T. Yao, N. Kobayashi, and T. Wasa, *Electroanalysis*, 3 (1991) 495-497.
2. J. A. Owens and J. S. Robinson, *J. Chromatogr.*, 307 (1984) 380-386.
3. B. Bleiberg, J. J. Steinberb, S. D. Katz, J. Wexler, and T. LeJemtel, *J. Chromatogr.*, 568 (1991) 301-308.
4. S. Ohmori, Y. Nose, H. Ogawa, K. Tsuyama, T. Hirota, H. Goto, Y. Yano, Y. Kondoh, K. Nakata, and S. Tsuboi, *J. Chromagr.*, 566 (1991) 1-8.
5. W. Rich, E. Johnson, L. Lois, P. Kabra, B. Stafford, and L. Marton, *Clin. Chem.*, 26 (1980) 1492-1498.
6. P. Chen, L. Nie, S. Yao, *J. Chromagr. B.*, 673 (1995) 153-158.