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A novel continuous subcutaneous lactate monitoring system☆

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Abstract

A novel continuous lactate monitoring system has been developed modifying the GlucoDay® portable medical device (A. Menarini Diagnostics), already present in the European market, and used to continuously measure glucose levels.

Lactate oxidase based biosensors have been developed immobilising the enzyme on nylon net and placing it on a Pt electrode. The biosensor was connected to the portable device provided with a micro-pump and coupled to a microdialysis system. It is capable to record subcutaneous lactate every 3 min.

In vitro analytical results confirmed that the sensors respond linearly in the interval of concentration between 0.1 and 10 mmol/L, covering the whole physiological range.

During prolonged monitoring periods, the response of the biosensors remained stable, showing a limited drift of 8%, within 60 h.

Stability tests are still on route. However, preliminary results have shown a shelf life of about 10 months.

In vivo experiments performed on healthy rabbits have demonstrated the good accuracy and reproducibility of the system. A correlation coefficient equal to 0.9547 ($N = 80$) was found, which represents a good correlation between the GlucoDay® and the laboratory reference analyser.

A 16 h in vivo monitoring on a healthy volunteer has been also performed.

Keywords: Biosensors; Continuous monitoring; Lactate; Microdialysis; Glucose

1. Introduction

Lactate is a substrate of great interest in clinical and sport medicine. Lactate levels are related to the status of anaerobic metabolism during muscle contraction, but several pathologic conditions can also cause increased lactate production. Healthy persons at rest have values roughly between 1 and 2 mmol/L, even if strenuous exercise increases these values. However, elevations in resting blood lactate concentration can be associated with survival risk. Elevated levels of lactate are, in fact, mainly found in conditions of hypoxia such as shock, hypovolumia, and left ventricular failure; conditions related to diseases such as diabetes mellitus, neoplasia, and liver disease; conditions associated with drugs or toxins such as ethanol, methanol, or salicylates (Oliver, 1970; Sacks, 1994).

In sports medicine, an understanding of lactate biochemistry can help to improve the aerobic endurance capacity in athletes. Lactate is formed when muscles use carbohydrates anaerobically for energy to support intensive or prolonged exercise. It increases sharply at a clearly defined point, termed the lactate threshold, which corresponds to the shift within the muscle cells to anaerobic metabolism. At this point, lactate is being produced faster than it can be

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metabolised and it accumulates. Elevated levels of blood lactate tend to lower blood pH with consequent disturbance of metabolism/protein structure, and beyond a certain tolerance level will result in muscle fatigue or 'cramping' (Karlson, 1986). Monitoring lactate levels is therefore an important tool for athletes in training, and the possibility to carry out this measurement in a continuous mode could enable them to follow lactate changes during training (Palleschi et al., 1990).

Continuous monitoring of metabolites using miniaturised portable instrumentation providing real-time measurements represents a significant advance in technology, especially when this monitoring is carried out in vivo. One way to perform in vivo continuous monitoring adopts a so-called "minimally invasive" method, which is to measure the concentration of different metabolites in the interstitial fluid of the skin using the microdialysis as sampling technique. This technique, introduced in the field of neuroscience in 1972 by Delgado (Delgado et al., 1972) and made popular by Ungerstedt (Ungerstedt et al., 1982; Ungerstedt, 1984; Tossman and Ungerstedt, 1986; Zetterstrom et al., 1984) uses a thin permeable dialysis membrane to construct a sampling probe, which is inserted under the skin and perfused by a physiological buffer. Permeable substances present in the interstitial fluid of the skin, and absent in the buffer, will diffuse down their concentration gradient across the membrane and directed into a suitable apparatus where the determination of the metabolite occurs.

Nowadays, it is quite frequent to couple microdialysis probes with biosensing detection modes, especially for the measurement of glucose in diabetic patients. This approach has been explored since nineties by several research groups (Keck et al., 1992; Meyerhoff et al., 1992, 1993a; Hashiguchi et al., 1994; Pfeiffer et al., 1993; Pfeiffer, 1994; Aalders et al., 1991; Jungheim et al., 2001).

In the past, we also have explored the use of the microdialysis technique measuring in vivo the subcutaneous glucose in healthy and diabetic patients, (Moscone and Mascini, 1992a, 1993; Moscone et al., 1992b) and, on the basis of the accumulated experience, a new wearable system, the GlucoDay® has been developed, produced and nowadays commercialised by A. Menarini Diagnostic (S.r.l. Firenze), and applied for the continuous glucose monitoring (Poscia et al., 2003). The system has been used for the subcutaneous monitoring of glucose continuously during 24 and 48 h with very little discomfort for the patient and high accuracy plus low imprecision of the results, giving additional information to the physicians to improve the disease treatment (Varalli et al., 2003; Maran et al., 2002).

In this paper, we extend the use of the GlucoDay® to allow the measurement of the subcutaneous lactate concentration, simply substituting the glucose biosensor with a lactate biosensor, optimising the performance of the whole system in order to fulfil the requirements of the already made portable instrument.

First attempts in this direction have been reported since 1993 by Meyerhoff et al., who measured lactate in whole blood employing a double lumen catheter and an amperometric lactate sensor located into a modified version of the Glucosensor Unitec Ulm wearable device (Meyerhoff et al., 1993b). However, long-term implant of catheters in vein are considered risky for the patients, so such kind of monitoring can be performed only in surgeries; in addition, the prototype device never reached the market.

De Boer et al. also carried out lactate monitoring in patients with shock (De Boer et al., 1994a) by subcutaneous microdialysis, but the dialysate was collected discontinuously and analysed later through an enzymatic fluorescent method. In addition, the same group proposed a transcutaneous microdialysis sampling in humans during exercise, performed by a heated aluminium diffusion chamber covered with a cellulose membrane, placed on stripped skin, and perfused with a physiological NaCl solution. However, they found a discrepancy between lactate levels in plasma and in transcutaneous dialysate, being the latter 6 times in excess. (De Boer et al., 1994b).

Our group already reported some in vitro, and then in vivo experiments on rabbits and also on a human volunteer who performed some physical exercise during the lactate monitoring (Volpe et al., 1995). However, in these experiments, monitoring lasted no longer than 4 h due to the bulkiness of the measuring system, which obliged the volunteer to stay near the laboratory bench.

More recently, other research groups (Yang et al., 1995; Pfeiffer et al., 1997), have carried out similar experiments following lactate changes in subcutaneous microdialysate of rats or small rodents, but they encountered different problems such as a half-life of the sensor of not more than 24 h, or a significant day to day variation in sensitivity (±50%). Moreover, their systems also involved bench apparatuses.

Some systems able to simultaneously measure glucose and lactate have been reported in the literature, always combined with microdialysis sampling probes (Wang et al., 2003; Jones et al., 2002; Yao et al., 2003; Perdomo et al., 2000; Petrou et al., 2002, 2003). However, in some cases these systems were coupled with flow injection systems that made the apparatuses quite bulky (requiring pumps, valves and reactors), so that they are not easily miniaturised and are suitable only for experiments on animals or during surgery where the patient is immobilised. In other cases, up to now devices have generally been shown to perform excellently in vitro, but have not been used yet for in vivo applications.

In this paper, the GlucoDay® modified for lactate measurement has been tested in vivo in rabbits and on a human volunteer. Lactate concentrations in the dialysate, measured every 3 min by the biosensor, and compared with venous blood samples (discontinuously collected throughout the experiments and assayed by the reference method) showed a good agreement. The lactate biosensor also showed a good linearity, a good operational stability with a limited drift of 8% within 60 h and a lifetime of about 10 months.

2. Materials and methods

2.1. Materials and equipment

Two types of L-Lactate oxidase enzymes (LOD) were used, one supplied by Sigma (Cat N L0638 from *Pediococcus* sp., 39 U/mg) and the other one by Roche (Cat N 11798197, *recombinant microorganism*, 55 U/mg). These enzymes were immobilised on nylon net discs (Ø = 4.5 cm, mesh = 120 cm^{-2}, thickness = 100 μm) from A. Bozzone, Appiano Gentile, Italy.

Polycarbonate membrane of 0.2 μm pore size was from Nucleopore (Pleasanton, CA); cellulose acetate membrane with 100 Da molecular weight cut off (MWCO) was prepared as previously reported (Mascini and Mazzei, 1987). For casting the cellulose acetate membrane, a precision gauge tool (from Precision Gauge and Tool Co., Dayton, OH) was used.

The GlucoDay® was manufactured by A. Menarini Diagnostics.

Electrochemical wall-jet cell from Metrohm (Switzerland), equipped with a Pt working electrode, an Ag/AgCl reference electrode and an Au auxiliary electrode was used for amperometric measurements. The applied potential was 650 mV (versus Ag/AgCl). Current was recorded with an L 250 E recorder from Linseis (Switzerland).

Miniplus 3 peristaltic pump was from Gilson Medical Electronics SA (France).

Two ways microdialysis probes (2.5 cm length) were supplied by A. Menarini Diagnostics, while one-way microdialysis probes (0.5 cm length) were supplied by MicroBiotech (Sweden).

2.2. Procedures

2.2.1. Enzyme immobilization

The nylon discs were immersed into a triethyloxonium tetrafluoroborate (TOTFB) solution (0.1 M in CH_2Cl_2). After washing with methanol, the discs were firstly put into polyethyleneimmine (PEI) solution (5% in distilled water), washed with water and then immersed into a glutaraldehyde solution (0.1 % in carbonate buffer pH = 10). After 1 h, several washing steps with phosphate buffer pH = 7.0 were carried out, then a lactate oxidase solution (40 mg in 2–3 mL of phosphate buffer pH 7.0) was spread onto the treated nylon net, and left at 4 °C for 12 h. Finally, the membrane was washed with a 0.1 mol/L glycine aqueous solution, with phosphate buffer and then stored in phosphate buffer (pH 7) at 4 °C. All the reagents used were supplied by Sigma.

2.2.2. Biosensor assembly

The lactate biosensors were assembled as already done for the glucose biosensors in the GlucoDay® medical device (Poscia et al., 2003). The sensor was a platinum anode (Ø = 0.4 mm) melted into a glass cylinder inserted into a silver tube, that works as a cathode. The electrode was covered by three membranes (a cellulose acetate membrane, the lactate oxidase membrane and a polycarbonate protective membrane), held in place by a small piece of teflon tube of suitable diameter. The small MWCO of the cellulose acetate membrane excludes the electrochemical interference, such as ascorbic and uric acid, allowing the passage of hydrogen peroxide only. Lactate oxidase was immobilised on nylon net, as indicated in the previous section.

The lactate control solution (0.5 mmol/L), used to check the functionality of the sensor, was prepared by dissolving L(+) lactate lithium salt (Sigma) and NaN_3 (Sigma) into a Dulbecco's physiological buffer.

In vitro experiments were carried out connecting the biosensors either to an amperometric biosensor detector, or to the GlucoDay® medical device (A. Menarini).

Linearity was evaluated connecting the biosensors to a microdialysis probe, which was introduced into Dulbecco buffered lactate solutions of different concentrations, ranging from 0.1 mmol/L to 12 mmol/L.

The lifetime measurements were conducted storing the LOD cells at 4 °C filled with Dulbecco's physiological buffer and checking the signal at fixed times, using a 0.5 mmol/L lactate standard solution.

In vivo experiments were carried out on four healthy female rabbits (7–8-weeks-old, of about 2 kg each), at the Research Toxicological Centre (RTC Pomezia, Italy). The lactate biosensors were connected to GlucoDay® instruments and to different types of microdialysis probes (two-ways and one-way probes). Six hours monitoring periods were recorded. Every 30 min, starting 2 h after the fiber insertion, venous blood samples were collected and both lactate and glucose concentrations were measured by the Biochemical Laboratory in the RTC centre.

3. Results and discussion

3.1. In vitro experiments

The analytical performances of five LOD based biosensors, coupled to microdialysis probes were evaluated.

Linearity was tested at flow rate of 13 μL/min on biosensors assembled with two types of LOD enzymes (Roche and Sigma) and connected to an amperometric detector. Fig. 1 shows the curves measured for both types of enzymes. The new based LOD biosensors respond linearly from 0.1 to 10 mmol/L lactate concentrations, covering the entire physiological range. Moreover, although their different sources, no significant differences between the two types of enzymes (Roche and Sigma) were observed, suggesting that both of them can be used.

The lifetime was measured storing the lactate biosensors at 4 °C. The response signals were checked periodically, using 1.0 mmol/L lactate solution at 30 μL/min, connecting the biosensors to a microdialysis probe and to an amperometric detector. The two groups of biosensors (one assembled with the Roche and the other with the Sigma enzyme) showed

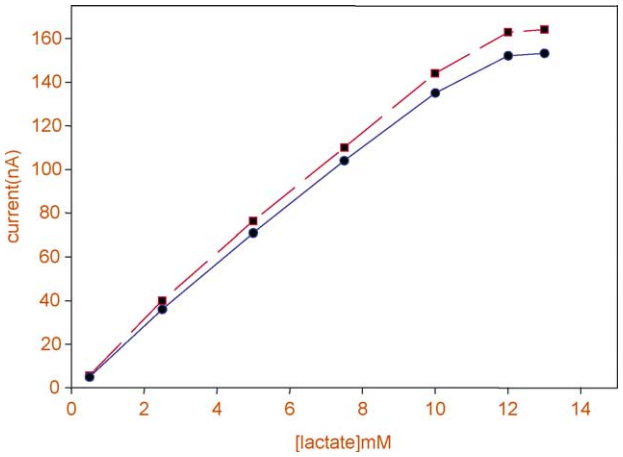

Fig. 1. Linearity curves of lactate for both enzyme at 13 μL/min. Dotted line: lactate oxidase from Roche; solid line: lactate oxidase from Sigma. Flow rate: 13 μL/min. Two ways microdialysis fiber.

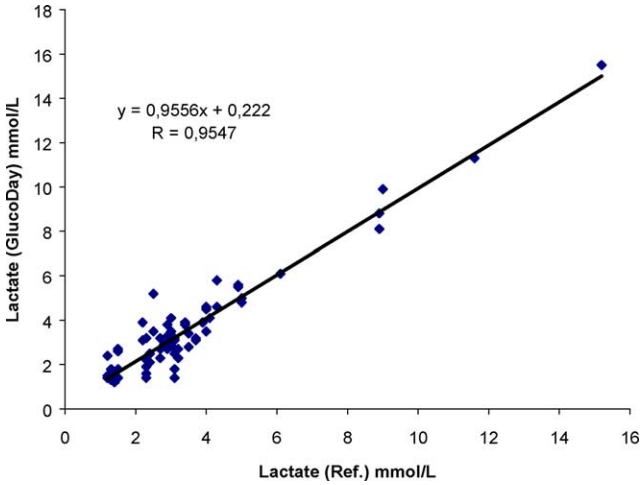

Fig. 2. Correlation curve ($N = 80$). Lactate concentration values obtained in vivo experiments with Glucoday (*Y*-axis) and correlated with values obtained with reference method (*X*-axis). Two ways microdialysis fiber.

about 30% decrement from the initial activity after 2 months. However, more prolonged stability study are still on route.

Possible interfering compounds, such as ascorbic or uric acid, do not interfere in the lactate measurements, because as it was previously demonstrated (Poscia et al., 2003) the acetate cellulose membrane acts as barrier towards such molecules, not allowing them to reach the electrode surface.

In vitro continuous monitoring experiments were performed pumping a 0.1 mmol/L buffered lactate solution at 13 μL/min flow rate, without connecting the microdialysis probe. After 60 h of data acquisition, 8% and 4% reduction of the biosensor response was observed, respectively for the Sigma and the Roche enzyme. These drifts are acceptable considering such a long period of time and confirm the high stability of these new biosensors.

3.2. *In vivo experiments*

Six hours continuos lactate monitoring experiments were performed on 4 healthy female rabbits. On each rabbit more than one GlucoDay® instrument was implanted, coupling different types of microdialysis probes (two-ways probe 2.5 cm length and one-way probe 0.5 cm length) and setting the flow rates at 13 μL/min.

Every 30 min, starting 2 h after the probe insertion, venous blood samples were collected from the left ear of each rabbit and immediately sent to the clinical laboratory to measure, both lactate and glucose concentrations.

The lactate concentrations, measured by a laboratory reference system, were compared to those measured by the GlucoDay®. A total of 80 data points were collected. The resulting correlation curve (Fig. 2) evidences the good accuracy of the system, in the whole physiological range ($N = 80$; $R = 0.9547$). The Bias error (mmol/L) was calculated as difference between individual results from the GlucoDay® and the mean of the laboratory reference values, and it was plotted versus the lactate concentration (Fig. 3). The graphic evidences, that more than 88% of data is within the ranges, indicated by the dotted lines (±1 mmol/L below 4 mmol/L and ±20% above 4 mmol/L that are limits very similar those fixed in an International Standard for glucose measurements).

Reproducibility studies were performed, measuring the subcutaneous lactate concentration on the same rabbit, using two independent GlucoDay® instruments and biosensors, both set at flow rates of 13 μL/min and connected to two-ways microdialysis probes. Fig. 4 reports the graphical behaviours of rabbit #7, which clearly indicates the good correlation between the two profiles ($N = 120$; $R = 0.9657$). Two independent instruments gave comparable results, when applied on the same rabbit.

Two different types of microdialysis probes were tested (one-way probe supplied by MicroBiotech and two-ways probe supplied by Menarini), working at constant flow rate (13 μL/min). Fig. 5 compares the resulting lactate profiles for rabbit #3. The two-ways probe, coupled to the GlucoDay® medical device, seems to measure lactate levels more accu-

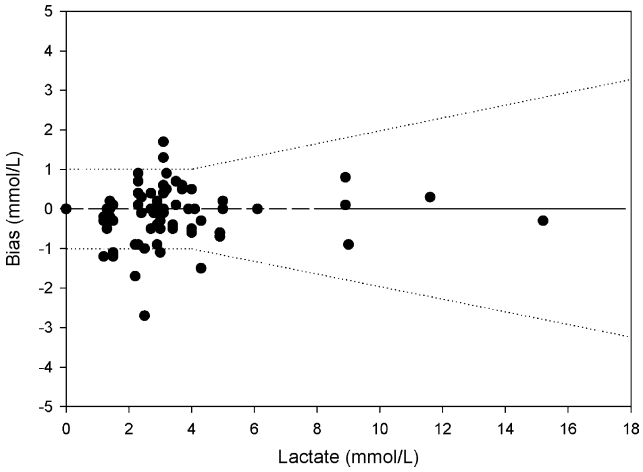

Fig. 3. Bias error (mmol/L) vs. lactate concentration (mmol/L).

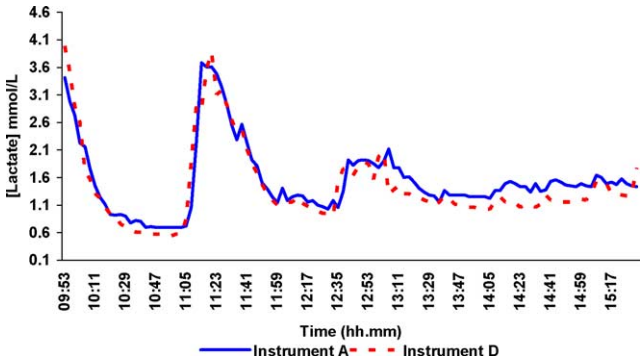

Fig. 4. Repeatability Study. Two GlucoDay instruments implanted on the same rabbit. Flow rate: 13 μL/min. Two ways microdialysis fiber.

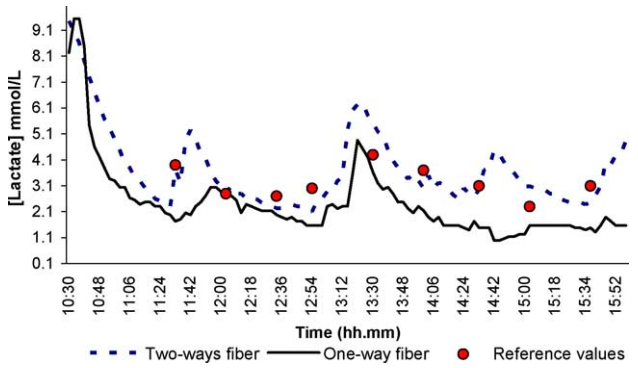

Fig. 5. Comparison of different types of microdialysis probes at 13 μL/min Both fibers were implanted in the same rabbit (#3).

rately than the one way probe. In fact, the average Bias % error for the two-ways probe is equal to 18%, whereas is nearly twice higher in case of the one-way probe. This is probably due to its better lactate recovery capability, which remains stable for the entire experiments. However, reducing the flow rate at 9 μL/min, the one-way probe improves its recovery capability, as it is clear from Fig. 6, in which the profiles obtained for rabbit #5 are reported. It must be noticed anyway that in case of the one-way probe, the signal is still more unstable compared to the two-ways probe.

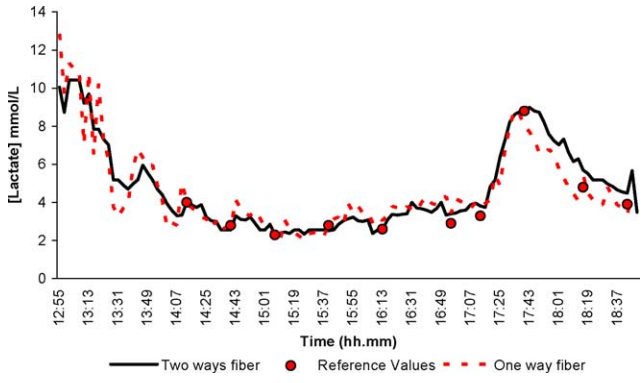

Fig. 6. Comparison between two types of fiber. One-way at flow rate 9 μL/min; Two-ways fiber at 13 μL/min. Both fibers were implanted in the same rabbit (#5).

During the in vivo monitoring, lactate infusions (90 μmol/mL L-lactic acid sodium salt solution at a rate of 30 μmol/kg/mL) were performed on rabbits #5 and #7, through a needle implanted on the right ear.

For comparison, infusions of only physiological solutions were performed on rabbits #1 and #3.

Fig. 7A and B show the profiles obtained with the GlucoDay® system in comparison with the venous blood samples. By looking at the profiles of rabbit #5 (Fig. 7A), following lactate infusion, an increment up to 9.1 mmol/L lactate was detected. However, when rabbit #1 (Fig. 7B) was infused only using physiological solution, the increment of lactate concentration was much higher, reaching 16.7 mmol/L, the same values found in the blood controls.

This could suggest that lactate infusions do not contribute significantly to variation in subcutaneous lactate concentrations. The lactate peaks observed in rabbit #1, may be attributed to "stress" physiological conditions. In fact, rabbit #1 appeared very stressed, during the entire experiment, evidencing lactate peaks nearly every time a blood sample was taken. However, without investigating more in detail the physiological causes of such lactate increments, the GlucoDay® coupled to the new lactate biosensors was able to detect promptly and accurately rapid changes in subcutaneous lactate levels.

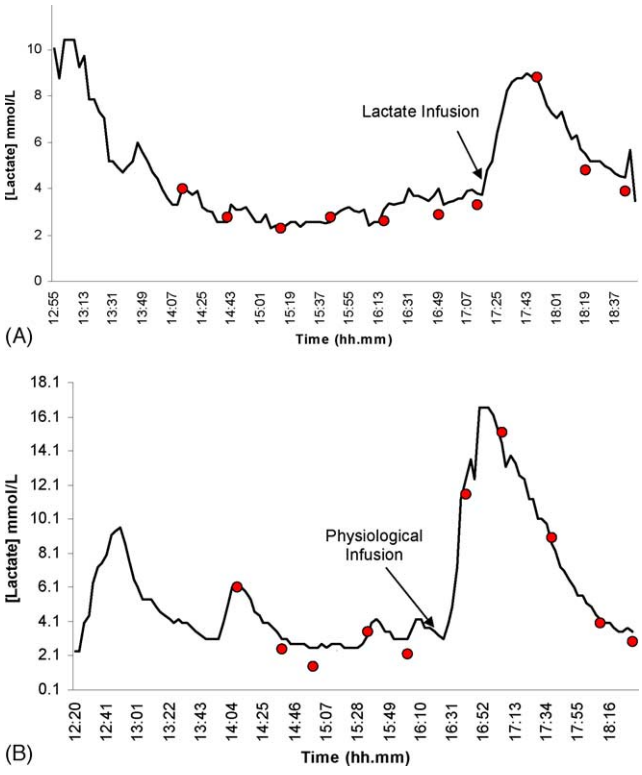

Fig. 7. (A) lactate infusion on Rabbit #5. Flow rate: 13 μL/min. Two ways microdialysis fiber. Arrow indicates the moment of the infusion of 90 μmol/mL of L-lactic acid at a rate of 30 μmol/kg/mL. (rabbit #5); (B) physiological infusion on rabbit #1. Flow rate: 13 μL/min. Two ways microdialysis fiber. Arrow indicates the moment of the infusion of a physiological solution (rabbit #1).

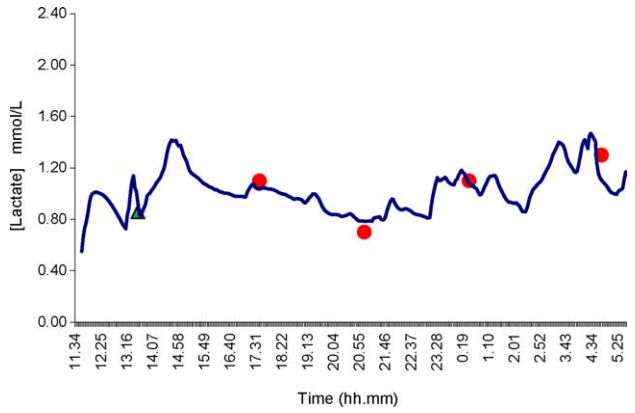

Fig. 8. Continuous monitoring of subcutaneous lactate in a human volunteer (male: 45 years old); (▲) calibration point; (●) blood venous sample measured by the reference laboratory instrument. Flow rate: 13 μL/min. Two ways microdialysis fiber.

Continuous monitoring of subcutaneous lactate was also performed in a human volunteer (male: 45 years old) in the Department of Internal Medicine of University of Padua (Italy) (Fig. 8). The monitoring was performed continuously for 16 h calibrating the instrument after 120 min from the insertion of the microdialysis probe into the subcutaneous tissue. The lactate values coming from the GlucoDay® was compared toward blood venous sample every 4 h. The correlation between the GlucoDay® measurements and the references values were in line with previous clinical data obtained for glucose monitoring on diabetic patients (Maran et al., 2002). After this preliminary result, we started a complete clinical trial on human volunteers, which is still in progress.

4. Conclusion

A novel continuous lactate monitoring system was developed modifying the GlucoDay® medical device (A. Menarini), already present in the European market, and used to continuously measure the glucose levels.

A lactate oxidase membrane was used in place of a glucose oxidase membrane, and the new cells were assembled in similar way to the GlucoDay® glucose sensors (Poscia et al., 2003).

The analytical performances of the new LOD based biosensors were deeply investigated. Their response was linear from 0.1 up to 10 mmol/L, an interval that surely covers the physiological range.

During prolonged monitoring periods the response of the LOD biosensors remained stable, showing acceptable drifts, not higher than 8%, within 60 h.

No interfering compounds, such as ascorbic or uric acid, affect the measurement of lactate. The acetate cellulose membrane acts in fact as barrier towards such a molecules, not allowing them to reach the electrode surface.

On the other hand, compared to the very high stability of GOD based biosensors; these new LOD sensors showed a lifetime of about 10 months.

In vivo experiments conducted on healthy rabbits confirmed the good accuracy of this new system ($N = 80$; $R = 0.9547$).

By comparing two different types of microdialytic probes, the results showed that the two-ways microdialysis probe recovers interstitial lactate better than the one-way probes, at the condition of the commercialised GlucoDay® (flow rate 13 μL/min). However, the recovery capability of the one-way probe, can be improved by reducing the flow rate. The GlucoDay® coupled to LOD biosensors was able to follow accurately rapid increment or decrement of lactate concentration.

A preliminary test conducted on human volunteers confirmed the feasibility of such measure in human subjects.

In conclusion, using the new LOD based biosensors in connection with the GlucoDay® medical device (A. Menarini), without any other modifications, the subcutaneous lactate concentration can be measured continuously (one data every 3 min). This opportunity opens a wide range of new applications, from clinical investigation to sport medicine.

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