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Reverse iontophoresis of L-lactate:

in vitro and *in vivo* studies.

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Abstract

This work investigates the reverse iontophoretic extraction of lactate, a widely used marker of tissue distress in critically ill patients and of sports performance. *In vitro* experiments were performed to establish the relationship between subdermal lactate levels and lactate iontophoretic extraction fluxes. Subsequently, the iontophoretic extraction of lactate was performed *in vivo* in healthy volunteers. Lactate was quickly and easily extracted by iontophoresis both *in vitro* and *in vivo*. During a short initial phase, iontophoresis extracts the lactate present in the skin reservoir, providing information of relevance, perhaps, for dermatological and cosmetic applications. In a second step, lactate is extracted from the interstitial subdermal fluid allowing local lactate kinetics to be followed in a completely non-invasive way. The simultaneous *in vivo* extraction of chloride, and its possible role as an internal standard to calibrate lactate reverse iontophoretic fluxes, was also demonstrated. Despite these positive findings, however, considerably more research is necessary to eliminate potential artefacts and to facilitate interpretation of the data.

Introduction

Lactic acid is a product of both anaerobic and aerobic metabolism[1]. Hyperlactatemia in critically ill patients occurs via different mechanisms. Under hypoxemic conditions, lactate levels increase disproportionately with pyruvate concentrations[2-4]; on the other hand, hyperlactatemia, with normal lactate to pyruvate ratios (10:1 to 15:1), is observed during the systemic stress response to severe infection or injury[2,4]. Generally speaking, lactate is considered an important, although unspecific, marker of tissue distress[4,5], and its levels are routinely measured in critical care units because of their prognostic significance, and to evaluate the success of therapeutic strategies[2,6-10]. Hyperlactatemia has also been associated with drug therapies involving nucleoside analogue reverse transcriptase inhibitors, epinephrine, metformin, phenformin[10-13], and in type I glycogen storage disease[14].

As well as these clinical applications, lactate is used as a marker of endurance and training efficiency[15-16]. The lactate threshold, the steady-state lactate (MLSS) and the maximal lactate steady-state workload (MLSSw) (i.e., workload that can be maintained over time without a continual blood lactate accumulation) are used to assess sports performance, and are based on serial measurements of systemic lactate levels[15-16]. In terms of lactate monitoring, a hand-held device (Accutrend Lactate[®]) has recently been introduced for sport and point-of-care applications[8,13], and active research on biosensors, microdialysis and micro-catheterization[17-19] is in progress. These different approaches are invasive, however, monitoring lactate either directly in the blood, or in the subcutaneous tissue and deeper organs, and require trained personnel. There exists, therefore, a rationale for the development of non-invasive technology, which can reduce the risk of infection, allow continuous monitoring, and offer a practical alternative for ambulatory care.

Reverse iontophoresis is a minimally invasive procedure which applies small electrical currents to the skin to enhance the transdermal transport of substances across the barrier[20]. The potential of this method has been demonstrated by the development of the GlucoWatch G2 Biographer[® 21] which can monitor glycemia, in a essentially continuous manner, for as long as 12 hours. While this device is not yet optimized, it is quite benign apart from the calibration procedure that requires a conventional finger-stick. Even this drawback may be addressed by a refinement of

the technique which uses an internal standard approach that can render the method completely non-invasive[22-23]. Indeed, this strategy has been successfully applied to predict the blood levels of lithium in bipolar patients being treated with this drug[24]. The internal standard procedure takes advantage of the fact that all ions present in the interstitial fluid are extracted with an efficiency that depends on their concentration and mobility. The internal standard is selected from the electrolytes present and its concentration is ideally high and relatively constant; i.e., its iontophoretic flux ($J_{I.S.}$) is effectively constant and can be used to normalize that of the analyte of interest ($J_{analyte}$) according to Eq.1:

$$J_{analyte} / J_{I.S.} = \gamma^{\#} . C_{analyte} \qquad \text{Equation 1}$$

where $\gamma^{\#}$ is the proportionality constant relating the ratio of iontophoretic extraction fluxes to the subdermal concentration of the analyte, $C_{analyte}$.

The objective of this work was to investigate the feasibility of non-invasively monitoring lactate by reverse iontophoresis. Lactic acid, a small and negatively charged compound at physiological pH (MW = 90.1, pK_a = 3.9), constitutes an excellent candidate for reverse iontophoretic extraction. L-lactate plasma concentrations in healthy adults fall in the range of 0.4-1.8 mM, increasing to 2-5 mM in severely injured patients, or during sport exercise[2,4,10,25].

In vitro experiments were first performed to examine the relationship between subdermal lactate levels and iontophoretic extraction fluxes. The specific aim was to validate a linear relationship between lactate transport ($J_{Lactate}$) and the subdermal concentration of the analyte ($C_{Lactate}$):

$$J_{Lactate} = \gamma. C_{Lactate} \qquad \text{Equation 2}$$

In Eq.2, γ is a constant which depends on the efficiency of the iontophoretic extraction of lactate. In a second step, the transdermal iontophoretic extraction of lactate was carried out *in vivo* in healthy volunteers. The simultaneous extraction of chloride as a possible internal standard for lactate extraction was also investigated.

Materials and Methods

Materials

Porcine ears were obtained fresh from an abattoir (Société d'Exploitation d'Abbatage, Annecy, France) and cleaned under cold running water. Skin was removed with a dermatome set to 750 µm (Zimmer™ Air Dermatome, Dover, Ohio) and cut into small squares (9 cm^2), which were wrapped individually in Parafilm™ and maintained at -20°C for no longer than one month before use.

Sodium chloride, L-lactate acid sodium salt, and HEPES buffer were obtained from Fluka (St Quentin Fallavier, France). Glycine-glycine; glycine-glycine hydrochloride, L-glutamic acid, glutamic pyruvic transaminase (L-alanine aminotransferase, ALAT) from porcine heart, L-lactic dehydrogenase type VIII (L-LDH) from chicken heart, and β-nicotinamide adenine dinucleotide were obtained from Sigma-Aldrich (St Quentin Fallavier, France) and Roche Diagnostics France (Meylan, France). De-ionized water (resistivity > 18 Mohm/cm^2) was used to prepare all solutions.

In vitro iontophoretic experiments.

The skin was mounted between the upper and lower parts of vertical iontophoresis cells[26]; the area available for transport was 0.73 cm^2. The anodal and cathodal compartments were filled with unbuffered 50 mM NaCl. The subdermal donor compartment was charged with 25 mM HEPES buffered at pH 7.4, containing 133 mM NaCl, to which different concentrations of L-lactate (0.5 − 4 mM) were added (n = 6). Direct current (0.4 mA) was applied for 5 hours via Ag/AgCl electrodes from a power supply (Kepco APH-1000DM, MB Electronique, France). The entire contents of the anodal compartment were removed every hour and analyzed for L-lactate using an enzymatic assay described below. In a control experiment, which investigated iontophoretic extraction of the endogenous L-lactate reservoir (n = 3), the subdermal solution contained no lactate whatsoever. A second, no-current, control experiment measured the passive flux of L-lactate from a 4 mM subdermal donor (n = 12).

In vivo experiments

The clinical protocol was approved by the internal review board of the Geneva University Cantonal Hospital. Informed consent was obtained from all participants (aged 25-40 years), who were normal and healthy, and who had no history of skin disease. Four subjects (A-D) participated on a single occasion, and a single volunteer participated twice (E1, E2). The volunteers were allowed to eat and drink during the experiments. Two glass chambers (2 cm^2) were fixed to the forearm (at a site which had been cleaned with an alcohol swab) using a Teflon ring and sealed with silicone grease. The ensemble was held firmly in place with medical tape (3M 9772L Foam Tape, 3M Healthcare, St Paul, MN, USA). The chambers were filled with 1.2 mL of unbuffered saline (15 mM); Ag/AgCl electrodes were then introduced and maintained at least 5 mm from the skin surface by means of a plastic lid that covered the cell. The outward flux of chloride was also measured in these experiments. This was easier to accomplish against a lower 'background' level of Cl$^-$ in the electrode chambers, explaining the use of 15 mM NaCl *in vivo* (as compared to 50 mM *in vitro*). A Phoresor II Auto (Model PM850, Iomed Inc., Salt Lake City, UT) delivered a constant, direct current of 0.6 mA (0.3 mA/cm^2) for 5 hours during which 20 samples were removed at intervals of 15 minutes. At the end of each interval, the current was stopped and the electrode solutions were completely removed and refreshed. The samples were subsequently analyzed for lactate and chloride by ion-chromatography (see below). After 1 hour, capillary blood lactatemia was measured at the beginning of each sampling cycle using a conventional blood lactate monitor (Accutrend Lactate[®], Roche Diagnostics, Mannheim, Germany).

Analytical chemistry

For the *in vitro* experiments, the enzymatic assay used for L-lactate was based on a modification of previously published methods[27-28], and involved the conversion of L-lactate to pyruvate by L-LDH and the associated reduction of NAD$^+$ to NADH. Pyruvate was consumed by transamination by ALAT to drive the reaction to NADH. Briefly, 200 µl of sample was mixed with 700 µl of glycine-glycine buffer (210 mM) and 100 µL of an aqueous solution of NAD in water (8 mg/mL). The glycine-glycine buffer (pH 10) contained 35 mM L-glutamate and the appropriate concentration of enzymes to provide 14 units of ALAT and 84 units L-LDH per sample. The reaction

was carried out at 25°C for 11 minutes, after which the amount of NADH formed was quantified by fluorescence (excitation = 340 nm, emission = 450 nm). A calibration curve was generated before each set of measurements.

For the *in vivo* samples, lactate and chloride were assayed by ion chromatography (Dionex ion chromatograph 600 system, Dionex, Sunnyvale, CA) equipped with a gradient pump (GP-50), a thermal compartment (AS-50) and an electrochemical detector (ED-50). Separation was accomplished on an anion column (AS-11) preceded by a guard column (AG-11) through which a 20 mM sodium hydroxide mobile phase was perfused. Quantification was performed in the suppressed conductivity mode; the electric current applied to the suppressor (ASRS 4) was 50 mA.

Data analysis and statistics

Lactate iontophoretic fluxes were directly calculated from the amount (moles) extracted in each collection interval normalized by the duration of the interval (hours). Chloride transport ($Q_{Cl,transported}$) was determined by mass balance:

$$Q_{Cl,final} = Q_{Cl,initial} - Q_{Cl,anode} + Q_{Cl,transported} \qquad \text{Equation 3}$$

where $Q_{Cl,final}$ and $Q_{Cl,initial}$ are the amounts of chloride present at the anodal solution at the end and beginning of a sampling interval, respectively. $Q_{Cl,anode}$ is the amount of chloride consumed at the anode during the sampling interval (t), that is:

$$Q_{Cl,anode} = I \cdot t / F \qquad \text{Equation 4}$$

where I is the current in Amperes (or Coulombs per second), and F is Faraday's constant (96,500 Coulombs). The transport number of an ion, which is the fraction of the total charge transported by this species, was calculated from Eq.5:

$$t_i = \frac{I}{z_i \cdot F \cdot J_i} \qquad \text{Equation 5}$$

where J_i is the iontophoretic extraction flux of the ion (moles/second), and z is the valence.

The efficiency of lactate extraction (γ) *in vitro,* was obtained by regression of the $J_{Lactate}$ data with respect to $C_{Lactate}$. *In vivo*, γ was determined at each sampling interval by dividing the lactate flux ($J_{Lactate}$) by the average lactate concentration in the blood over the sampling interval (i.e., the mean of the capillary blood concentrations measured at the beginning and end of each sampling period):

$$\gamma = \frac{J_{Lactate}}{c_{blood}} = \frac{J_{Lactate}}{\left(c_{initial} + c_{final}\right)/2} \qquad\qquad \text{Equation 6}$$

where C_{blood} is the averaged lactatemia that corresponds to the iontophoretic interval during which a flux $J_{Lactate}$ is measured.

In vivo lactate fluxes are plotted at the mid-point of the sampling interval, while blood concentrations are shown at the actual time of measurement. Data are expressed as mean ± standard deviation (SD). Statistical tests and linear regression analysis were performed with Graph Pad Prism 3.02 software (San Diego, CA). The significance of the linear regressions was tested by the corresponding ANOVAs. The level of statistical significance was fixed at $p \leq 0.05$.

Results and Discussion

The *in vitro* experiments demonstrated that L-lactate iontophoretic extraction fluxes were easily measurable and increased proportionally with the subdermal concentration of the analyte (Figures 1 and 2). The correlation between $J_{Lactate}$ and $C_{Lactate}$, however, was acceptable only after current had been applied for 2 hours, with the coefficient of determination, r^2 (which indicates the proportion of the total variation in the flux that is explained by the fitted regression) being quite low until the third interval of extraction (Table 1). The poor linearity observed during the initial phase of extraction is probably due to an endogenous reservoir of L-lactate in pig-skin. To confirm this hypothesis, a control experiment was performed in which no lactate was introduced into the subdermal solution. The fact that appreciable lactate was nevertheless extracted to the anode (Figure 1) confirmed that lactate must have been stored in the skin. In fact, in this control study, the iontophoretic extraction flux of lactate during the first hour of current passage was approximately equal to that measured when the subdermal solution contained up to 2 mM lactate. Skin reservoirs for glucose[29], urea[30] and (in bipolar patients taking the drug) lithium[24] have been previously observed. L-lactate accumulates in the skin as a product of glycolysis[31] and, at a lower level, from sweating[32-34]. Furthermore, additional lactate accumulation in excised pig ear skin may be due to post-mortem glycolysis[35-36]. It follows that these *in vitro* experiments may overestimate the magnitude of the lactate reservoir *in vivo*. Finally, it should be noticed that passive lactate fluxes were 9.6±16.3 and 6.4±12.4 nmol/h after 2 and 6 hours of transport, respectively: this means that iontophoresis not only enhanced significantly the transdermal flux of lactate, but that it also accelerated the rate at which the reservoir of compound was cleared from the skin.

Once endogenous lactate had been emptied from the skin, the iontophoretic flux became progressively more dependent on the subdermal concentration, (as shown by higher values of r^2 in Table 1). From the third hour of extraction, the pooled value of γ (Eq.2) was 9.3 $\mu l.h^{-1}$ (23 $\mu l.h^{-1}.mA^{-1}$), which represents the efficiency of the lactate extraction process (Figure 2, Table 1). For lithium, γ has been reported to be 42 $\mu l.h^{-1}$ (105 $\mu l.h^{-1}.mA^{-1}$)[37], consistent with a higher efficiency of extraction for this smaller cation. In contrast, for phenytoin, a larger and only partially (12%) charged anion, γ was only 0.84 $\mu l/h$ (2.1 $\mu l.h^{-1}.mA^{-1}$)[38]. The transport number of lactate was

calculated to be 0.13% for the 0.5 mM subdermal solution, increasing to 0.34% at 4 mM. Lactate competes with other anions present in the subdermal compartment, and with cations (e.g., Na^+) being "delivered" from the anode, to transport the charge across the skin. Obviously, lactate competes better as its concentration increases, allowing a bigger fraction of charge flowing to be carried by the analyte.

The second phase of this work explored the feasibility of iontophoretically extracting lactate *in vivo*, in humans (Figure 3). It was demonstrated right away that lactate was easily extractable and quantifiable even with relatively short sampling intervals of only 15 minutes (Figure 3). This confirmed that lactate is a good candidate for reverse iontophoretic sampling, and the high efficiency of the process suggested that lactate kinetics may be followed non-invasively. It was further observed that a significant skin reservoir of lactate was present in all individuals studied, and lactate fluxes were high in the first sampling periods. As previously mentioned, lactate is known to accumulate in the skin[31-34]; indeed, NMR has been used to suggest that lactate represents ~10% of water-extractable materials of human stratum corneum[34], and 25% of its ionic composition. Lactate extraction during the first sampling interval was 184 ± 95 nmol/cm^2, a value in good agreement with that obtained by extraction of the analyte from stratum corneum (209 nmol/cm^2 [33]). It follows that the initial, short periods of iontophoresis can provide "local" information about lactate and other components of the skin's natural moisturizing factor[34] that may be useful for dermatological and cosmetic purposes.

Once the skin reservoir had been depleted, the reverse iontophoretic fluxes of lactate could be compared with capillary blood levels. In previous work, good agreement between the plasma levels of both lithium and glucose and their respective extraction fluxes[23,24,30] have been observed, indicating a relatively fast equilibrium between systemic and subdermal interstitial fluid levels for these two compounds. As a result, the efficiency of extraction could be estimated from the blood levels. On the contrary, it has been shown that subcutaneous, arterial, venous and capillary concentrations of lactate are not always in equilibrium[39-41], and this is reinforced by the results in Figures 3 and 4. In some cases (C and E1) iontophoretic extraction followed blood levels, and the apparent efficiencies of extraction (γ) were relatively constant throughout the experiment (Figure 4):- subject C: $\gamma = 73 \pm 6.3$ $\mu l.h^{-1}$; subject

E1: $\gamma = 51 \pm 11$ $\mu l.h^{-1}$. However, in the other volunteers, γ was very high during some of the sampling intervals, especially those towards the end of the experiment (Table 2). It has been reported that subcutaneous adipocytes release lactate both in the post-absorptive state and under hyper-insulinemia conditions[39-41]. Postprandial concentrations of lactate in subcutaneous tissue are significantly higher that the corresponding arterial and plasma values[39-41]. Because the subjects were allowed to eat and drink during this preliminary *in vivo* study, the high iontophoretic fluxes (and apparent efficiencies of extraction) may be the result of high postprandial concentrations of lactate in the subcutaneous interstitial fluid subsequent to the ingestion of nourishment. Thus, while, the data in Figures 3 and 4 support the feasibility of lactate monitoring by reverse iontophoresis *in vivo*, additional work is clearly needed to better control for potential artefacts and to facilitate interpretation of the results.

Finally, because reverse iontophoresis is non-specific, it was possible to measure, using the same *in vivo* samples, the extraction of chloride as well; these unique results are in Table 2. The deduced Cl^- transport number was $44 \pm 8\%$, a value in reasonable agreement with that measured previously *in vitro* and indicative of the skin's cation permselectivity[42]. The constancy of the experimental chloride fluxes suggests that this anion may be a useful internal standard with which to normalize the iontophoretic extraction of negatively-charged compounds, in the same way that sodium has been used for cations[24]. However, the use of Ag/AgCl electrodes imposes some limitations; first, a theoretical estimate of the amount of Cl^- consumed at the anode must be determined and, second, chloride must already be present in the anode solution to participate in the electrode reaction. In other words, the chloride transported through the skin is extracted into a solution that already contains a certain amount of chloride. Needless to say, this imposes an analytical challenge in that the change in the Cl^- content of the anode solution must be sufficiently large to be reliably quantified. Generally speaking, in the experiments reported here, this was the case; however, the occasionally observed aberrant values are a signal that the use of Cl^- as an effective internal standard will require careful optimization.

Conclusions

This work demonstrates, *in vitro* and *in vivo*, that lactate can be quickly and easily extracted across the skin by reverse iontophoresis. In a short initial phase, iontophoresis extracts the lactate present within a skin depot, providing information of potential interest in dermatological and cosmetic applications. Subsequently, lactate is transported from the subdermal interstitial fluid allowing lactate kinetics to be followed in a completely non-invasive way. Considerably more research is necessary, however, to fully exploit the potential benefits of the approach in an ambiguous manner.

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Figure legends

Figure 1: *In vitro* iontophoretic extraction fluxes of lactate as a function of sampling time and lactate subdermal concentration. In order to facilitate visualization of the data, the symbols corresponding to the 0.5 and 1 mM subdermal donors have been slightly displaced.

Figure 2: Correlations between lactate iontophoretic fluxes and the subdermal concentration of the analyte after 3, 4 and 5 hours of current passage. The regression analyses of the data are in Table 1.

Figure 3: Capillary blood lactate profiles and reverse iontophoretically extracted lactate in five volunteers. Subject E participated on two occasions (panels E1 and E2).

Figure 4: Evolution of the apparent extraction efficiency of lactate (γ) *in vivo* as a function of time.

Table 1: Linear regressions of the data in Figure 2 according to the equation: $J_{Lactate} = \gamma \bullet C_{Lactate} + Intercept$. $J_{Lactate}$ is the anodal extraction flux of lactate in nanomoles/hour, and $C_{Lactate}$ is its subdermal concentration in mM. All the regressions were significant ($p<0.001$). The mean values ($\pm95\%$ confidence of interval) for the slope and the intercept are shown.

Time (hr)	γ (μL.h^{-1})	Intercept (nmol.h^{-1})	r^2
1	7.2 ± 3.5	31.4 ± 7.9	0.34
2	8.9 ± 2.2	18.3 ± 5.1	0.69
3*	9.4 ± 1.7	13.1 ± 3.9	0.78
4*	9.4 ± 1.7	11.6 ± 4.0	0.80
5*	9.0 ± 1.4	10.8 ± 3.2	0.83
Pooled values*	9.3	11.8	

*Pooled values from the results at 3, 4 and 5 hours.

Table 2: *In vivo* extraction fluxes of chloride (μmoles/hour)

Subject	A	B	C	D	E.1	E.2
Mean	9.55	9.10	8.02	8.92	9.93	12.64
S.D.	0.70	0.95	1.17	3.39	0.57	1.50
C.V. (%)	7.3	10.4	14.6	38.1	5.8	11.9
n*	18	16	16	16	15	14

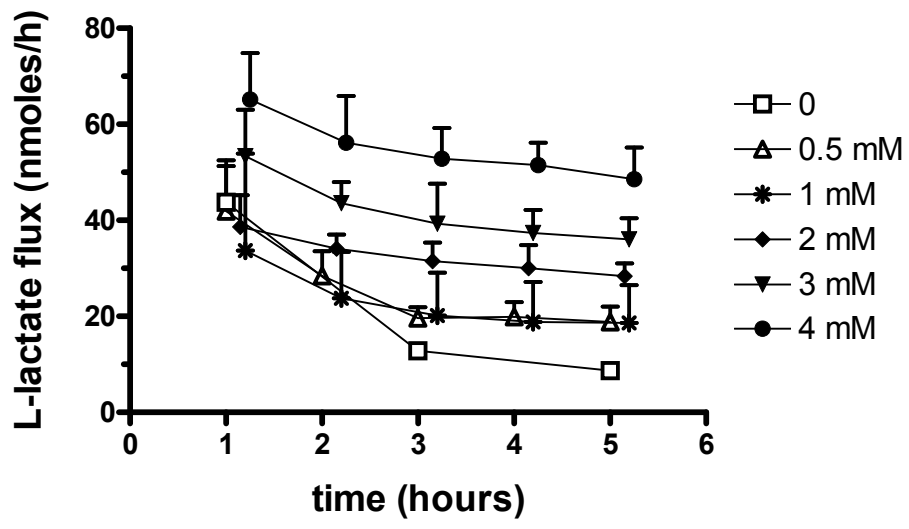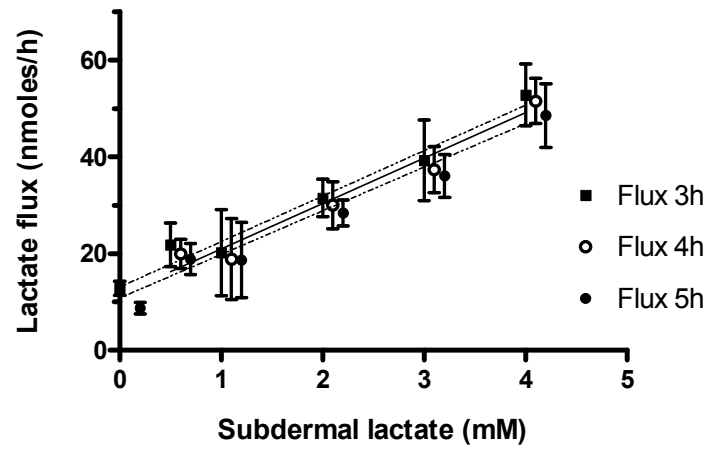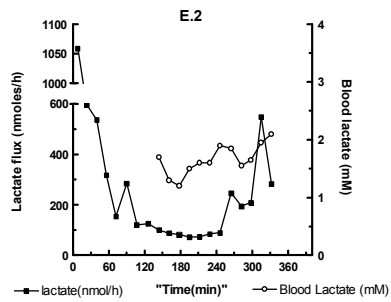* The total number of sampling intervals (n) was 20. Missing fluxes were aberrant values as determined by a Dixon test ($p < 0.95$).

Figure 1

Figure 2

Figure 3

Figure 4