Recovery of Endogenous Ions from Subcutaneous and Intramuscular Spaces in Horses Using Ultrafiltrate Probes

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The objective of this study was to determine if a novel ultrafiltrate probe could be used to sample endogenous ions from subcutaneous and intramuscular interstitial fluid in horses. The second objective was to determine if the concentrations of the recovered ions were physiologically relevant. This was done by determining if the concentration of one of the ions changed in response to intravenous infusion. It was concluded that sodium and potassium equilibrate rapidly between the vascular and interstitial space and that interstitial fluid concentrations of these ions reflect the plasma concentrations. Calcium concentrations, however, differed according to site, indicating that factors other than diffusion play a role in determining calcium concentrations in interstitial fluids.

Ultrafiltration, like microdialysis, is a process which extracts small molecules and ions through semi-permeable membranes. The chief difference between them is the mechanism by which solutes are transported. In microdialysis, the concentration gradient of the solute is the driving force in the net rate of transfer, while in ultrafiltration a pressure differential is the driving force.

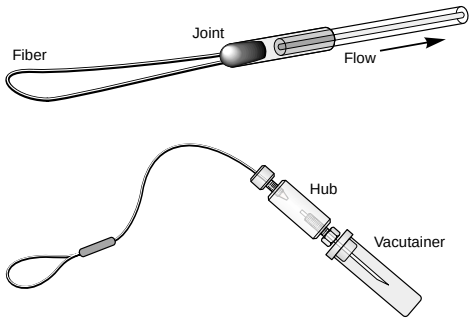
Both processes are similar to hemodialysis (1). In hemodialysis, the process of diffusion is used to transport solutes. The nature and physical properties of the semi-permeable membranes determine the molecular weight and chemical properties of the substances able to pass through and those excluded. Therefore, water and many small solutes will pass through these membranes with both processes. Diffusion will be facilitated by a steep concentration gradient, increased molecular motion, small size, and lack of protein binding of molecules on one side of the membrane and increased membrane surface area. Ultrafiltration rate is influenced by the transmembrane pressure gradient, and water being pushed (or pulled) through the membrane will be accompanied by small solutes in the approximate concentrations as on the other side of the membrane.

Recently, both techniques have been used in bioanalytical chemistry as sampling tools in vivo (2-8). Because of the differences between microdialysis and ultrafiltration, each has advantages that can make one more useful or appropriate for specific situations. In microdialysis, where the driving force is diffusion, conditions can be adjusted so that there is minimal fluid loss and minimal analyte removed from the tissue. For this reason, microdialysis probes have been used in brain neurochemistry studies in rats (2) and in humans (3).

With ultrafiltration, there is a net loss of fluid from the tissue, including both water and solutes in their original concentrations. Ultrafiltration (UF) probes have been employed in vivo in metabolite and pharmacokinetic studies to provide continuous sampling of the subcutaneous interstitial space in rats (4), dogs (5), mice (6), cats (7), and humans (8). The molecules studied have included inorganic electrolytes, such as sodium, potassium, calcium and inorganic phosphorus, as well as glucose, urea, creatinine, and antibiotic drugs. Ultrafiltration probes offer many useful features in the collection of interstitial fluid. Fluid is collected continuously, the samples require little or no preparation prior to analysis, the animals can move freely, and the sampling can continue for long periods of time (days to weeks), thus permitting multiple experiments on each subject with minimal stress.

F1

The ultrafiltration assembly.



We report here the results of experiments using UF probes to study sodium, potassium, and calcium concentrations in the subcutaneous and intramuscular interstitial fluids of horses.

Materials and Methods

The ultrafiltration assembly ***(F1)*** consists of the semi-permeable membrane fibers attached to a piece of connecting tubing that inserts into a hub assembly with a needle that can be inserted into a Vacutainer™ tube (Monoject, Sherwood Medical, St. Louis, MO). The vacuum in the tube provides the pressure differential which is the driving force for the filtration.

Five healthy, adult horses, aged 3-15 years and weighing 400-500 kg, were used in this study. They were allowed free choice water and fed either grass or alfalfa hay *ad libidum*. The horses were sedated with 5 mg detomidine hydrochloride (Dormosedan, SmithKline Beecham, West Chester, PA) intravenously. The two insertion sites used were the lateral neck and the lateral upper foreleg. The sites were shaved, surgically prepared with betadine and alcohol and anesthetized with 2% lidocaine. Two stab incisions were made with a #15 scalpel blade approximately 8 cm apart. A 10 gauge needle was introduced through one incision, tunneled under the skin and exited through the second incision. The ultrafiltration probe was drawn through the needle by the connecting tubing until the ultrafiltration membrane portion was in the desired location. The tubing was sutured to the skin with non-absorbable suture and cyanoacrylate glue was applied. A collecting needle and hub assembly were attached to the tubing and suction was provided by either a 10 or 15 ml Vacutainer™ tube. The flow rate was approximately 1 µl/min with either vacuum tube size.

Implantation of Ultrafiltration Probes Intramuscularly

The procedure was similar to the subcutaneous placement. After sterile preparation and local anesthesia of the skin, a 10 gauge needle was placed through the extensor carpi radialis muscle. The probe was drawn into the needle and the needle withdrawn leaving the semi-permeable membrane within the muscle belly. In addition, the outlet tubing was coiled in a subcutaneous pocket and sutured to the muscle fascia in order to minimize tension on the tubing caused by animal movement. The tubing was passed through a stab incision in the skin and secured to the skin by non-absorbable sutures, and the Vacutainer™/hub assembly placed in the pouch which was affixed to the medial foreleg by elastic adhesive bandages. Surgical removal of the probes after a series of experiments on an animal verified that the placement of the probe membrane had remained intramuscular.

Horses tolerated the placement and wearing of the probes very well and were able to move freely around the stall at all times except during the brief periods that exogenous substances were being infused. The animals did not need to be restrained to change the Vacutainer™ tubes, and were able to walk, eat, drink and lie down at will. Ultrafiltration probes were implanted subcutaneously and intramuscularly in opposite legs, thus allowing simultaneous sampling from multiple sites.

Infusion of Calcium Chloride

The horses were given 28 g of calcium chloride (Lyphomed, Deerfield, IL) diluted in 2 liters of 0.9% sterile saline and infused within 100 minutes via a 14 gauge, 5½ inch catheter (Abbocath, Abbott Laboratories, North Chicago, IL) placed in the jugular vein and removed after infusion. Samples of blood were taken by venipuncture from the opposite jugular vein, collected in microfuge tubes with lithium heparin and centrifuged to separate the plasma. Samples of the interstitial fluid were taken by removing the Vacutainer™ tube and replacing it with a new tube, thus maintaining negative pressure on the probe to allow ultrafiltration to continue. Samples were taken every 30 minutes over at least 2 hours to establish a baseline before infusion was begun, and then every 30 or 40 minutes thereafter, for at least 4 hours after the beginning of the infusion. Thus, the blood samples represented a point in time, whereas the subcutaneous and intramuscular ultrafiltrate samples represent a time average. For the calcium determinations, an ultrafiltrate of the plasma was also examined. Immediately after the blood was centrifuged, an ultrafiltrate probe was placed in the plasma and vacuum applied to initiate filtration.

Analysis of Samples

The sodium and potassium concentrations were measured with an ion selective electrode (Fisher Scientific, Pittsburgh, PA). The ion meters were calibrated with 2000 ppm and 150 ppm standard solutions immediately before each set of samples was measured. The samples, both lithium

T1

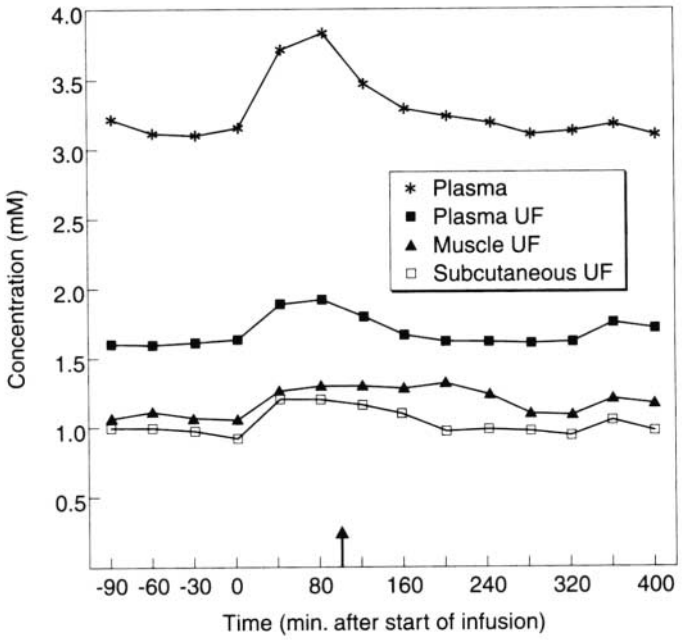
Baseline sodium, potassium and calcium levels in different tissues (mean ±SD).

Electrolyte	Plasma UF	Subcutaneous UF	Muscle UF
Sodium	140.1 ± 2.4 (5)*	137.4 ± 1.5 (4)	139.8 ± 2.2 (3)
Potassium	3.86 ± 0.08 (5)	3.95 ± 0.07 (4)	3.92 ± 0.03 (3)
Calcium	1.63 ± 0.08 (4)	0.99 ± 0.04 (3)	1.14 ± 0.15 (3)

**Number in parentheses is number of different animals used for that site.*

F2

Calcium concentrations after intravenous calcium infusion (averaged). Arrow indicates time infusion was finished. Ultrafiltrate is abbreviated as UF.



heparinized plasma and ultrafiltrates, were diluted to provide enough volume for accurate measurement at a concentration in the linear range of the meter. Double distilled deionized water was used that gave a reading of 0 ppm when tested with the ion selective electrodes. To measure sodium, samples were diluted by a factor of twenty by adding 190 ml water to 10 ml of sample. Fifty microliters of sample were diluted to 200 ml total volume to measure potassium. The concentration was read from the digital display 15 seconds after the electrode was immersed in the sample and converted to the molar concentration.

Calcium concentrations were determined spectophotometricly at 575 nm based on the absorption maximum of the calcium-cresolphthalein complexone complex using reagent kit number 587 from Sigma Diagnostics (Sigma Diagnostics, St. Louis, MO). A Varian Series 634 spectrophotometer was used with reagent mixture in the reference cell for all measurements. The reagent mixture was prepared by combining equal volumes of calcium binding reagent and calcium buffer immediately before use. Two milliliters of this mixture were pipetted and mixed with 20 ml of either heparinized plasma, ultrafiltrate or standard solution. A calibration curve was generated before each set of measurements by measuring the absorbance of standard solution at 5, 10, and 15 mg/dL. The calcium concentration of samples was then determined by relating the measured absorbance to the linear relationship of the calibration curve.

Statistical Methods

There were four possible samples per horse: the plasma, an ultrafiltrate of the plasma, subcutaneous and intramuscular interstitial fluid ultrafiltrate. Not every site was sampled on every animal, but paired samples of plasma and at least one interstitial ultrafiltrate site were taken from each animal. Statistical analyses were performed using repeated measures ANOVA if three sampling sites were measured, and paired t-test if two sampling sites were measured. The confidence level was set to 95%.

Results

Since there was no significant difference between subcutaneous neck and leg values and a small number of horses had the probe implanted in the neck, those data were pooled as subcutaneous. There was no significant difference in sodium and potassium concentration by site of sampling (plasma, subcutaneous, muscle) within or between animals (***T1***).

In all subjects, calcium concentrations in the plasma and in the ultrafiltrate of the plasma were both significantly higher than either the subcutaneous or muscle interstitial fluid. In general, the concentration of calcium in the subcutaneous ultrafiltrate was lower than that in the muscle, but the difference was not always statistically significant.

When calcium was infused, the total calcium and ultrafiltrate of the plasma calcium concentrations remained elevated above baseline for 70-110 min. after end of infusion (***F2***). The subcutaneous ultrafiltrate calcium concentration was elevated above baseline for an additional 30-40 minutes. The intramuscular ultrafiltrate calcium concentration was elevated above baseline for an additional 150-160 minutes compared to plasma. The range in times given is due to differences in sampling times between animals.

Discussion

The objective in these experiments was to study sodium, potassium, and calcium in subcutaneous and intramuscular interstitial fluid and to compare these values with the plasma concentrations. In addition,

we infused calcium intravenously and monitored the concentrations over time in these three spaces. This was done to demonstrate that we could detect physiological changes in calcium with this technique, and was in some ways a complimentary study to Linhares and Kissinger (4), in which an ultrafiltration probe was used to monitor insulin-induced potassium dynamics in the subcutaneous interstitial space.

The total body fluid in any mammalian species is divided between the extracellular and intracellular fluid (9,10). The intracellular fluid is the larger compartment. The extracellular fluid is further divided into the blood plasma, interstitial and transcellular fluid. The interstitial fluid space is the largest of these three. Although there is exchange of water and small ions by diffusion across membranes between all of these spaces, there are powerful mechanisms, e.g., the sodium potassium pump, that maintain ion gradients and thus different concentrations of many of these small ions in each of these spaces. For example, intracellular concentrations of sodium and calcium are very low and of potassium are very high, as compared to extracellular concentrations of these solutes (9-12).

In the horse, the principal repository of potassium is in muscle tissue, comprising 75% of the total body potassium (13). The majority (51%) of sodium in the horse is in the skeleton; only 10.8% is in the muscle (13). Chronic depletion of potassium is corrected by reducing stores in the skeleton and muscle, whereas the alimentary sodium, normally 12.4% of total body sodium, is preferentially tapped to make up sodium deficiencies. Exercise can also cause temporary shifts in relative concentrations of electrolytes in the extracellular and intracellular spaces (14-17). Alterations in ion concentrations have also been documented in certain disease conditions, such as exertional rhabdomyolysis (18), hyperkalemic periodic paralysis in the horse (19) or in man (20), inappropriate ADH Syndrome (21), and as a side effect of some drugs, e.g., diuretics (22).

One of the questions addressed in ultrafiltration studies has been whether the ultrafiltrate fluid reflects normal interstitial fluid. It is not known if the implantation of the probe causes alteration in the components of the space. The interstitial space is usually under negative pressure (12). We observed no significant local edema after the first 24-48 hours after implantation, and the values for sodium and potassium did not vary significantly over the course of the experiments, which lasted up to two weeks. Thus, we concluded that edema was not changing the concentrations of the ions studied. Previous experiments in rats (4) demonstrated that the subcutaneous UF concentrations of sodium, potassium, calcium, and inorganic phosphorous corresponded well with literature values of these ions. Our results using horses also correlate well with reported interstitial values for the ions we measured (10,11).

Another question we wished to address was whether the intramuscular interstitial fluid concentrations of sodium, potassium and calcium differed from the subcutaneous. One problem of muscle physiological studies involves the difficulties in making measurements of the various small ions and molecules at the site of interest. For example, excitation-contraction coupling involves calcium release and the subsequent sodium intracellular influx with depolarization and the later potassium efflux with repolarization. Thus the steady state composition of the interstitial fluid in a muscle could potentially be quite different from that of the subcutaneous interstitial space and the plasma space.

Various techniques that have been used to study either a steady state or the influence of exercise on the concentrations of certain muscle constituents in horses and cattle include muscle biopsy (23,24) and arteriovenous differences (25). Most often examined are the fiber composition of the muscle, oxidative and glycolytic enzyme activities, and energy substrates and metabolites. Relatively little attention has been given to measuring the concentrations of electrolytes in situ, despite some evidence that release of potassium ions from muscle tissue may be involved with the development of fatigue (26). We believe that measuring ultrafiltrate of muscle may prove to be a useful adjunct to these techniques, particularly in cases where blood constituent concentrations may not reflect changes in the muscle.

These data suggest that sodium and potassium equilibrate rapidly between the vascular and interstitial space and that interstitial fluid concentrations of sodium and potassium reflect the plasma concentration. There was no statistically significant difference between the subcutaneous and intramuscular interstitial concentrations of these ions.

There was, however, a significant difference between the plasma UF and the interstitial concentrations of calcium, with the plasma always being higher. And although the calcium concentration of the subcutaneous ultrafiltrate was generally lower than the muscle ultrafiltrate, the difference was not always statistically significant. One possible explanation for this is that the concentration of calcium in the intramuscular microenvironment is higher due to the constant influx and efflux of calcium in excitation-contraction coupling. Other possible reasons for this could be changes in capillary pore sizes of the two tissues allowing different amounts of calcium to leave the vascular space, or different ratios of ionized vs. bound calcium in the muscle vs. subcutaneous interstitium. Because of the ultrafiltration process, the calcium detected would be the ionized and complexed fractions, and therefore the most important physiologically (21,27,28). In the plasma, around 50 per cent of the total calcium is in the ionized form, with the remainder bound either to plasma proteins (about 40 per cent) or com-

T2

Feeding alfalfa vs. grass hay: comparison of average calcium concentrations obtained from different sites.

Calcium (mM) ± SD				
Hay	**Plasma**	**Plasma UF**	**Subcutaneous UF**	**Muscle UF**
Alfalfa	3.28 ± 0.87	2.12 ± 0.87	1.82 ± 0.7	1.3 ± 0.18
Grass	3.04 ± 0.47	1.64 ± 0.16	1.02 ± 0.10	1.16 ± 0.17
p value	0.068	0.007	< 0.001	0.001

plexed in an nonionized way (about 10 per cent) with anions such as citrate or phosphate (21).

When calcium was infused, the plasma UF, subcutaneous UF, and muscle UF were all elevated at 40 and 80 minutes after the beginning of infusion ***(F2)***. Although the plasma UF and subcutaneous calcium UF levels had begun to decline by t=120 min, the muscle UF remained elevated until t=200 min and did not return to baseline values till t=280 min, 80 minutes after the other two. We concluded that this was reflective of calcium leaving the intramuscular interstitial space more slowly than the subcutaneous interstitial space.

Calcium levels were higher when horses were fed alfalfa than grass hay ***(T2)***. The calcium content of the hays was not analyzed and, since the animals were fed free choice, the precise amount of hay eaten was not known. In general, alfalfa hay can be expected to have 0.5-2.0 % calcium, while most grass hays have calcium concentrations of 0.3-0.6 % (dry basis) (29).

In these experiments we were able to demonstrate that ultrafiltration probes implanted in subcutaneous and intramuscular spaces of horses were useful in sampling the interstitial fluid of these sites over periods of times up to two weeks without hampering the free movement of the animals. Further, the values obtained for sodium, potassium, and calcium from these places reflect true changes in concentrations of these electrolytes in these spaces. We conclude that ultrafiltration probes are another useful tool that can be used in physiologic and metabolic studies.

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