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New Method for Automating Sample Collection from *In Vivo* Ultrafiltration Probes

A new technique is described for continuous collection of ultrafiltrates from freely-moving rats, mice and other small rodents. During ultrafiltration, a vacuum simultaneously withdraws and filters the extracellular fluid surrounding the implanted probe. A disposable needle for the HoneyComb fraction collector connects a standard laboratory vacuum pump, an ultrafiltration probe and the sample vial. When capped vials are placed in the fraction collector, each one of them becomes a mini-vacutainer as soon as the needle pierces the cap. This approach permits collection of small-volume samples, eliminates potential contamination or binding associated with peristaltic tubing, and removes the need to disturb the animals through exchange of conventional vacutainers. In vitro *applications are also discussed, as well as use of this technique to evaluate the flow rate of saliva in the human mouth.*

Introduction

Ultrafiltration is a membrane-based sampling technique with both advantages and disadvantages relative to microdialysis, as listed in ***T1***. Sample collection from ultrafiltration probes requires a vacuum, forcing the surrounding fluid first to pass through pores in the semi-permeable membrane which filter out macromolecules. The sampled fluid then travels along a collection tube and is ultimately deposited in a collection vessel, which can also be the source of the vacuum (e.g. a vacutainer). Large animals (dogs, sheep, horses, cows, humans) can carry a vacutainer and retain freedom of movement. Smaller animals such as mice, rats and other rodents won't accommodate such a burden and instead are housed in a cage with the vacutainer attached to a tether, or the collection tubing is externalized to a vacuum source outside the cage.

Peristaltic pumps have also been used as a vacuum source, allowing ultrafiltrate to be deposited continuously into fraction collector vials. However, this approach requires the ultrafiltrate to pass through the peristaltic tubing on the way to the vial. Peristaltic tubing is formulated to be soft and pliable, but such characteristics come from the use of plasticizing chemicals which can contaminate fluid washing through the tube. Given the increasingly sensitive analytical methods used to chase the low concentrations of analyte (e.g. unbound drug) common to ultrafiltrates, such contamination could create a significant interference in the assay. Analyte from the ultrafiltrate may also bind to this tubing. If ultrafiltration is used to assess changes in the availability of fluid from a particular sample, the flow from the probe should be as consistent as possible. However, the flow characteristics of a peristaltic pump will change over time as the peristaltic tubing stretches. For these reasons, we sought an alternative method of continuous collection from an ultrafiltration probe to minimize or eliminate such problems.

Materials and Methods

A new "vacuum needle" (p/n MF-7016, BASi, West Lafayette, Indiana) was created for the HoneyComb Refrigerated Fraction Collector (BASi), as illustrated in ***F1***. This collector is a standard component in the Culex® automated blood sampler as well as in microdialysis systems manufactured by BASi. Needles manufactured for fraction collectors and autosampling devices often have an outer sleeve which serves as a vent when the septum of a capped vial is initially pierced. This vent ("air-bleed cannula") relieves pressure as fluid is deposited or withdrawn from a sealed vial. We created a needle in which the vent was extended so that it could be attached to a vacuum source. This meant that once the cap was pierced by the needle, all air within the vial would be withdrawn, creating a vacuum within that vial as long as the needle remained in place. The vacuum source was a standard laboratory vacuum pump (model DOA-P184-AA, Gast Manufacturing, Benton Harbor, Michigan).

For *in vitro* studies, or collection of saliva, the probe (model UF-3-2 or UF-3-12, BASi) was wetted either by soaking for an hour or more in distilled water, or rinsing under running tap water for one minute. For *in vivo* studies, the probe was implanted dry using the standard procedure outlined in the probe manual. The animal was allowed to recover from anesthesia and surgery at least overnight, and this was sufficient time for the probe to become wetted by the surrounding tissue and fluids.

To prepare for sampling, the probe tubing was attached to the central port of the vacuum needle. The other port was connected to the vacuum. Standard borosilicate glass vials (p/n MF-5270, BASi) were capped with snap-on plastic caps (p/n MF-5273, BASi) and labeled with chads (p/n CX-1800B, BASi) before being loaded into the collector. The vacuum pump was started at 10 to 15 in Hg (inches of mercury) and the fraction collector was set to the desired number of vials and collection interval (5 to 90 min). During flow rate studies, capped vials were preweighed before use and reweighed after ultrafiltrate collection.

Results and Discussion

Flow Experiments

During initial tests, we assumed that a crimp cap seal would be required to create the tightest possible seal on the vial. For comparison, snap cap vials were also tested on the same system. Under the same conditions, there was appreciably more fluid in the vials with the snap caps, as illustrated in ***F2***. Snap caps were also easier to remove when transferring samples for analysis and were selected for all subsequent studies.

The simple pump used in this study adjusted the vacuum by means of a valve and an analog gauge. Increasing the vacuum increased the flow rate from the probe when it was immersed in a beaker of water (***F3***). Eventually, a point was reached in which the increase in flow was not linear due to the prevalence of gas bubbles associated with outgassing from the sample and the connecting tubing. Studies conducted *in vivo* also showed considerable outgassing when the vacuum exceeded 12 in Hg, but the limiting factor appeared to be availability of fluid in the tissue sampled by the probe.

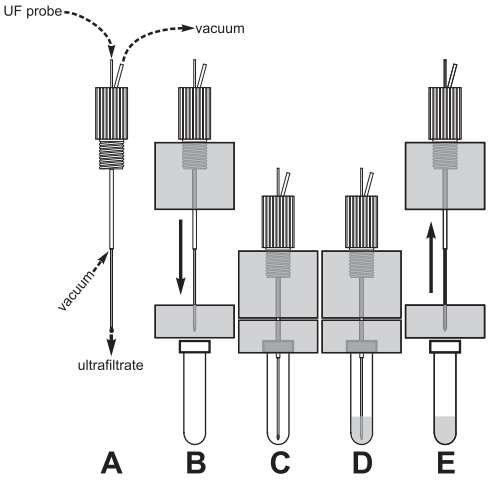
Multiple Probe Experiments

Rather than dedicating a vacuum pump for each probe, an arrangement was made to share the pump by using a trap,

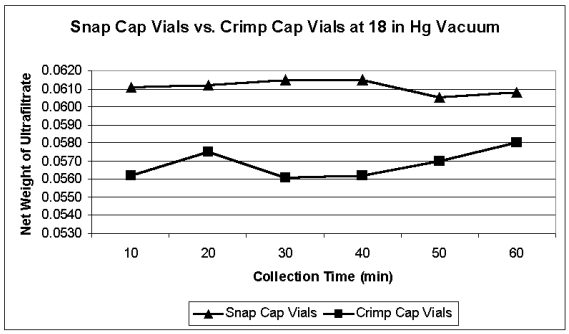
T1. Comparison of membrane sampling techniques.

Feature	Ultrafiltration	Microdialysis
Semi-permeable membrane.	Yes. Typically 20 - 360 mm.	Yes. Typically 1 - 10 mm.
Flow depends on membrane surface area.	Yes. The larger the surface area the more flow from the probe.	No. Flow is controlled by syringe pump which perfuses probe.
Membrane molecular weight cutoff (MWCO) ~ 30,000 Da.	Yes.	Yes.
Samples are protein-free.	Depending on how a protein is defined - molecules larger than the MWCO are excluded.	Molecules even smaller than the MWCO are excluded. The practical limit is 5,000 Da.
Fluid collected is the actual extracellular fluid (ECF).	No. Each sample is a filtrate of the extracellular fluid, but it is only filtered and not diluted. The concentration of low molecular weight analytes should be the same.	No. Sample is a dialysate of the extracellular fluid so the concentration of low molecular weight analytes will be lower than the extracellular fluid.
Fluid balance within the tissue is maintained.	No. The extracellular fluid is removed and must be replenished by fluid from blood capillaries nearby.	Yes. Fluid is neither added nor removed by the probe.
Concentration of analyte in ECF can be determined.	Yes. Since the ultrafiltrate is just filtered ECF.	Concentration can be estimated through additional studies in which another analyte is added to the dialysis perfusion fluid and its transfer into the tissue is determined.
Sample represents what?	Average concentration of analyte in the ECF over the entire sampling interval.	Average concentration of some percentage of analyte in the ECF over the entire sampling interval.
Does sample volume reflect tissue perfusion?	Yes. Flow will increase or decrease according to the amount of fluid available, including replenishment of ECF by blood flow to the tissue. This can be affected by temperature, anesthesia, sleep and fear.	No. Probe is being continuously perfused by the system so the sample volume should be consistent in each vial regardless of local blood flow.

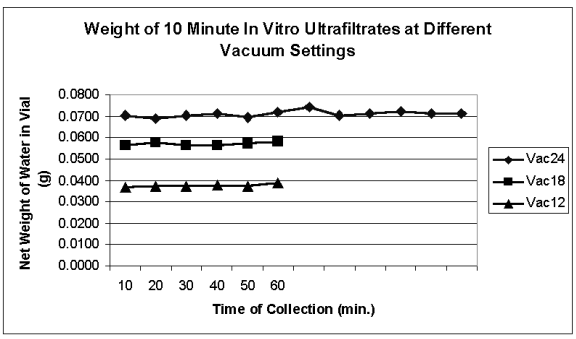
F1. The vacuum needle (A) has a central needle connected via a needle port to the ultrafiltration probe. It also has a vacuum port connected to the cannula surrounding the needle. The needle is inserted into the needle holder on the fraction collector which guides it in and out of the collection vials (B). When the needle is fully inserted into the vial (C) all air in the vial is removed by the external vacuum pump attached to the needle. Ultrafiltrate fluid then flows in the vial (D) until the needle is removed from the vial and the vacuum is then interrupted (E). The transition time between vials is approximately two seconds.



F2. Although both crimp cap and snap cap vials can be used, the snap cap vials maintain a better seal. In this study, the two types of vials were loaded into the same collector connected to the same probe and vacuum pump. The probe was fully wetted and immersed in water. Under identical conditions, more fluid was collected in the vials with snap caps.



F3. The pump was adjusted to increase or decrease the vacuum. An increase in vacuum to 24 in Hg (Vac24) increased the rate of ultrafiltrate collection, while a lower vacuum level of 12 in Hg (Vac 12) decreased the collection rate. However, the increase in yield from increased vacuum was not linear. Higher vacuum increased outgassing. In this situation, the amount of fluid available for ultrafiltration was virtually infinite since the probe was in a beaker of water.



as illustrated in ***F4***. This had the additional benefit of protecting the pump in case the vial was overfilled during the experiment. Since it was also desirable to conserve equipment, two vacuum needles were placed in the same collector so that collections from two probes could be conducted simultaneously, as illustrated in ***F4*** and ***F5***.

In Vivo vs. In Vitro Flow Rates

When UF-3-2 probes were immersed in water, volumes of 50-60 µL were readily obtained during 10 minute collection periods. When the same probes were placed in the biceps femoris muscle of the rat, comparable volumes would require collection periods up to at least two hours, depending on the time of day. ***T2*** illustrates day vs. night differences in ultrafiltrate volumes collected from UF-3-2 probes in the muscle tissue of four Sprague-Dawley male rats, collected over periods of several hours in a vacutainer. ***F6*** illustrates the flow from a subcutaneous UF-3-2 probe in another rat using the new vacuum needle and more frequent samples. Flow rates vary according to how "juicy" the tissue is during the collection period. *In vivo* ultrafiltration removes the extracellular fluid (ECF) from tissue surrounding the probe membrane. This ECF is replaced by fluid from blood capillaries, so ultrafiltration flow rates are strongly influenced by this process. If blood is flowing slowly to the tissue being sampled, the flow rate from the probe will likewise be slow. If blood flow increases, the flow rate from the probe should likewise increase. For example, we've noted that rats with subcutaneous probes produce more ultrafiltrate overnight when they are active than during the day when they are mostly sleeping. An anesthetized rat produces practically no ultrafiltrate, while the same rat produces plenty once it wakes up and starts drinking, eating and moving around.

Carbamazepine in Subcutaneous Ultrafiltrates

F7 provides a comparison between

plasma and ultrafiltrate concentrations for carbamazepine (CBZ) and its epoxide metabolite in the same rat during the same time period. This study was conducted during continuous intravenous infusion of CBZ over an extended period to establish a steady state concentration of drug. During the same period of time, 7 blood samples were collected vs. 13 ultrafiltrate samples, both of which showed a steady state concentration of CBZ. The mean concentration in the ultrafiltrates (85 ng/mL) was slightly less than half the mean concentration in the plasma (190 ng/mL).

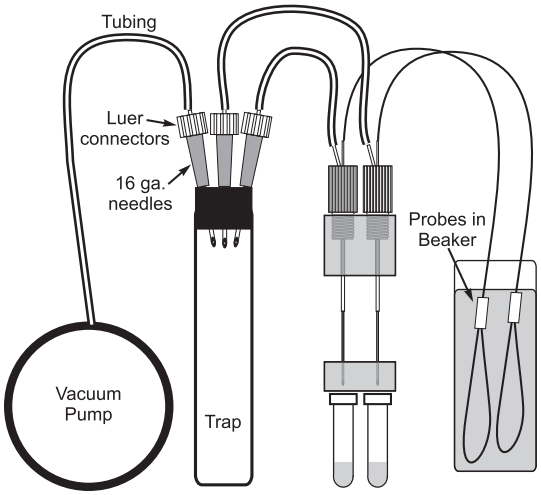
Saliva Collection

In a study to evaluate the ability of the method to measure changes in human saliva flow rate, the membrane portion of a UF-3-12 probe was positioned deep into the pocket between the lower lip and the lower gum. This is also the location of the duct from the submaxillary gland which produces approximately 75% of saliva in the mouth. The tubing from the probe was externalized and connected to the vacuum needle. Samples were collected every five minutes. A change in saliva flow was induced by placing a "Sweet-Tart" candy made from citric acid and dextrose on the tongue. ***F8*** illustrates the salivary flow rate before and after introduction of the candy.

Conclusions

The vacuum needle accessory is viable for continuous collection of ultrafiltrates from probes implanted into awake and freely-moving rodents. Ultrafiltrate sample collection is particularly attractive in rodents where there are limits to the amount of blood that can be removed. Prohibitions on blood collection pertain to the loss of erythrocytes. There is no such loss of cells in ultrafiltration. Therefore, there is virtually a limitless amount of ultrafiltrate available. Ultrafiltrates represent "unbound" drug concentrations and provide useful comparisons with plasma, as in ***F7***. Since ultrafiltrate flow *in vivo* is

F4. Creation of a trap allowed one vacuum pump to be shared by the two needles in the same fraction collector. The trap was made with materials readily available in the lab such as a large vacutainer, 16 gauge needles, luer connectors and tubing.

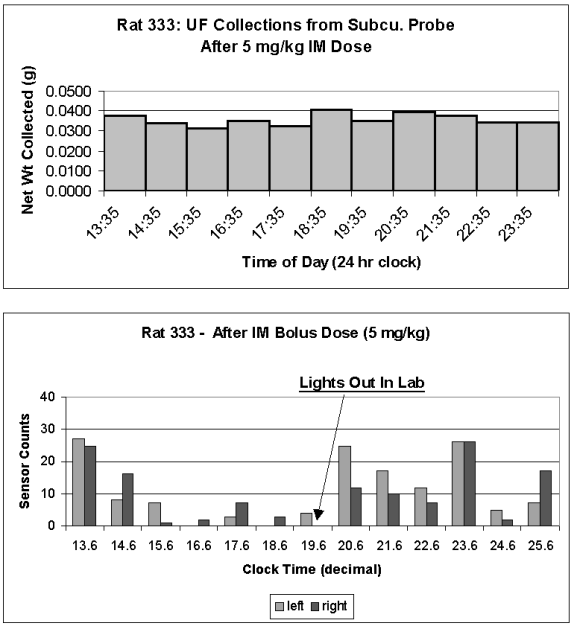


F5. Two vacuum needles installed in the HoneyComb fraction collector, each attached to the vacuum line. This arrangement collects samples from two probes in one animal, or one probe in each of two animals.

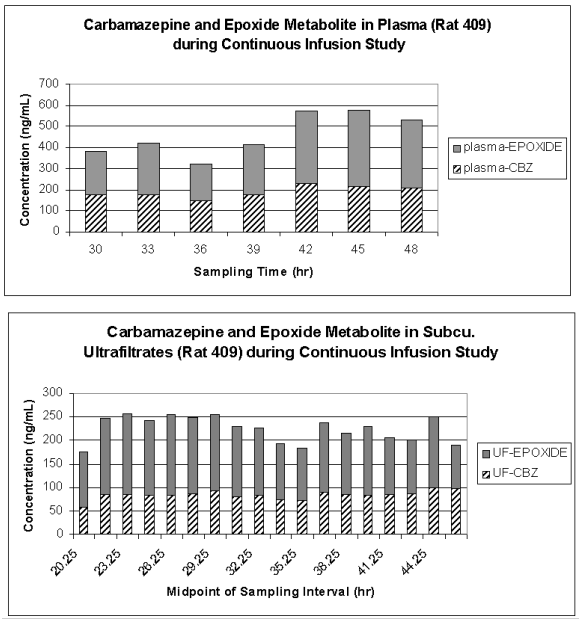
T2. Day-Night Flow Rates (µL/hr) of Ultrafiltrates from Muscle Tissue: UF-3-2 Probes in Four Sprague-Dawley Males (400-450 g).

Rat	9 a.m.	1 p.m.	5 p.m.	10 p.m.	9 a.m.
2	18.3	19.3	19.6	19.6	20.8
3	21.0	14.3	16.8	14.8	20.0
4	20.3	18.2	19.0	19.5	14.0
5	20.3	21.0	20.8	23.8	11.3

F6. Rat 333 was housed in a BASi Raturn Caging System which recorded rotational behavior (bottom graph) while simultaneously collecting ultrafiltrate (top graph) using the new vacuum needle technique.



F7. Comparison of Carbamazepine and its Epoxide Metabolite in the Plasma and Subcutaneous Ultrafiltrate of the Same Rat During the Same Time Period. The purpose of the study was to establish a steady state concentration of the drug during continuous infusion. This is demonstrated in both samples, with the concentration of both the drug and metabolites in the ultrafiltrates being less than half the plasma concentration.



dependent upon the availability of the extracellular fluid and blood flow within the tissue, this technique should be explored further as a way to indirectly monitor regional blood flow to specific tissues.

The vacuum needle approach shows promise as a non-invasive technique for human pharmacokinetics and pharmacodynamics via collection of saliva samples from humans. Since the probe can be disconnected from the vacuum needle quickly and then transferred over to a needle hub for vacutainer collections, it would be possible to start with frequent sampling at the fraction collector and then switch to longer sampling periods using the vacutainers. This approach would be most useful when collecting early samples to define drug absorption after oral dosing. One example of a pharmacodynamic application would be the change in salivary output relative to a drug dose, a study in which a precise and automated method of salivary sample collection could be very useful.

One objective of using this approach with animals was to minimize the stress associated with human presence. The noise of the pump itself is worth considering as a source of stress. The diaphragm-driven vacuum pumps can be particularly loud. However, we suspect that since the pump noise is continuous, it may be less aggravating than an intermittent noise. Use of a trap, as illustrated in ***F4***, enables us to move the pump to another room and diminish the noise considerably.

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F8. A UF-3-12 ultrafiltration probe was placed in the mouth of a human volunteer and connected to the vacuum needle. Saliva was continuously sampled with the sample vials advanced automatically every 5 minutes. After 25 minutes, a citric acid/dextrose candy was placed on the tongue to stimulate saliva flow. An increase in saliva flow was noticed immediately by the subject and demonstrated by the data. This increased flow continued as long as the stimulant remained on the tongue.