

# The Microdialysis Shunt Probe: Profile of Analytes in Rats with Erratic Bile Flow or Rapid Changes in Analyte Concentration in the Bile

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The purpose of this study was to show the usefulness of the microdialysis shunt probe in experiments where there is either erratic bile flow or rapid changes in concentration of analyte in the bile. During studies in anesthetized rats with the bile flow diverted, it was observed that the bile flow rate can be erratic and that concentration changes in the bile can occur rapidly. It was demonstrated that (a) the *in vitro* recovery of analytes is independent of the shunt flow at rates  $>5 \mu\text{L}/\text{min}$ ; (b) the recovery is independent of stop/start flow behavior in the shunt and will only decrease if the shunt flow is stagnant for long periods of time (i.e.,  $>15 \text{ min}$ ); and (c) the recovery of analyte through the probe tracks rapid changes in concentration and does not reflect changes in bile flow through the shunt. The implications of these *in vitro* studies on probe calibration and *in vivo* measurements are discussed.

The shunt microdialysis probe enables the monitoring of the concentration of low molecular weight species in the bile of awake, freely moving rats (1-3). A diagram of the shunt microdialysis probe is shown in **F1**. The shunt, which carries the bile flow, is of appropriate dimensions for implanting into the bile duct of an adult rat. Suspended inside

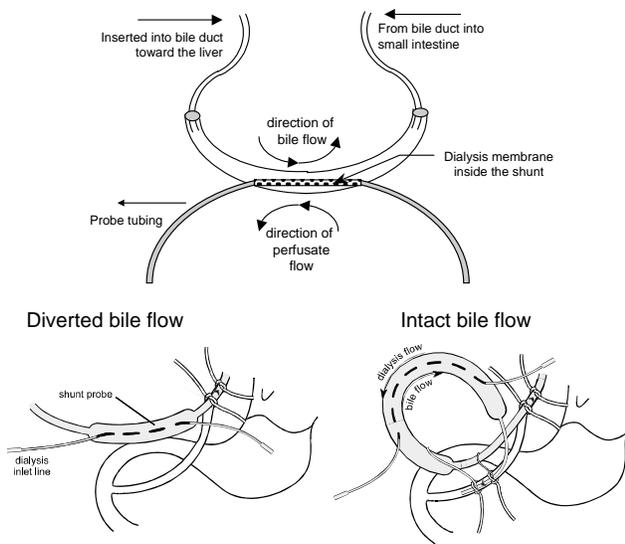
probe that continuously samples low molecular weight compounds from the bile. In anesthetized rats, the probe can be inserted at the upstream end (toward the liver) and bile collected (diverted flow, **F1**). The diverted flow experiment allows determination of the bile flow rate, measurement of the total bile collected and total dose eliminated, calibration of the microdialysis probe

and comparison of the concentration levels of the analyte to a rat whose bile flow is intact. When the shunt is inserted in both the upstream and downstream ends of the bile duct, the animal's bile flow is maintained (intact flow, **F1**). This configuration allows monitoring of the bile in an awake, freely moving rat (2).

In previous studies with the shunt probe, the ability to monitor phenolphthalein glucuronide in anesthetized animals with diverted and intact bile flow was demonstrated (4). Phenolphthalein glucuronide (PTG) is rapidly formed after administration of phenolphthalein (PT). This compound is typical of species that are conjugated and recirculated in the bile through enterohepatic cycling (5-8). It was also shown that the bile salts impact the microdialysis experiment. Perfusing the probe without bile salts results in loss of sample volume to the shunt via osmosis (9). The bile salts can also interact with the analyte and have an influence on the ability of the analyte to cross the

## F1

Diagram of the microdialysis shunt probe with implantation for diverted and intact bile flow.



microdialysis membrane (10). In this experiment, a solution of 2% bile salts in Ringer's was used as the perfusate to eliminate the effects of bile salts mentioned.

In the *in vivo* experiments involving diverted bile in anesthetized rats, bile flow measurements have varied greatly. In some studies the observed bile flow was uniform and in others it was erratic, sometimes exhibiting stop-start behavior. Reported bile flow in rodents ranges from 50-90  $\mu\text{L}/\text{min}/\text{kg}$  (11). Studies in rats indicate that anesthesia, body temperature, endogenous compounds such as bile acids, administration of drugs and hydrostatic pressure (8,11) can markedly influence bile flow.

Research from other laboratories has shown that the microdialysis response time is rapid, allowing on-line studies with temporal resolution of 1-2 minutes (12-14). Rapid equilibration would be expected in the shunt probe, as the shunt provides a hydrodynamic environment where the rate limiting step should be diffusion through the membrane and not through the biological fluid. The on-line studies in this experiment were done in 6-minute increments. This time period is more than adequate to allow for a steady state to establish at the microdialysis membrane.

Since the bile flow and analyte concentration can vary rapidly, it is important to know the response of the shunt probe to these changes and how these changes can affect the probe calibration and *in vivo* results. In this study several *in vitro* experiments were performed to mimic *in vivo* extremes in both bile flow and concentration and to evaluate the performance of the probe under these conditions. The ability of the probe to track concentration changes *in vivo* in the diverted, anesthetized rat is demonstrated in a rat exhibiting an erratic flow pattern.

## Experimental Procedures

### Reagents and solutions

The following reagents were used as obtained from Sigma Chemical Co. (St. Louis, MO): phenolphthalein, phenolphthalein glucuronic acid (sodium salt), bile salts (approximately 50% sodium cholate and 50% deoxycholate, Sigma # B-8756), KCl,  $\text{CaCl}_2$ , and NaCl. Ringer's solution was prepared from NaCl (155 mM), KCl (5.5 mM), and  $\text{CaCl}_2$  (2.3 mM). Bile salt Ringer's (BSR) solution was prepared by dissolving 2 g bile salts in 100 mL of Ringer's solution. HPLC grade acetonitrile and methanol, and reagent grade phosphoric acid and ammonium hydroxide for preparation of ammonium phosphate buffer, were obtained from Fisher Scientific (Pittsburgh, PA). Mobile phase was prepared by mixing solvents and buffer (volume:volume) followed by filtering through a 0.22 micron nylon filter under vacuum. All solutions were made using NANOpure (Barnstead Co., Boston, MA) deionized water. All chemicals were reagent grade or better and used as received.

### Analytical method

PTG was analyzed on-line using a reverse phase liquid chromatography (LC) method with UV detection. The analytical column was a C-18, ODS-3 phase II, 3 micron, 10 cm x 3.2 mm (BAS, MF-6213) with matching guard column (BAS, MF-6206) and a 0.5 micron in-line filter. The column was in an oven at 37 °C. The mobile phase was 10% ACN, 26% MeOH and 64% 50 mM ammonium phosphate buffer at pH=2.5. The mobile phase flow rate was 0.8 mL/min, resulting in an approximate retention time for PTG of 3.4 minutes. A Shimadzu UV-Vis SPD-6AV detector was used at 230 nm with data output to an analog-to-digital converter and data analysis with BAS ChromGraph software. The injection system consisted of a CMA-160 valve with a CMA-100 microinjection syringe pump/con-

troller. The injector was equipped with a 3- $\mu\text{L}$  injection loop. On-line injections of dialysates were performed every 6 min, with a 6 sec injection loop flush onto the column. The perfusate flow set at 1  $\mu\text{L}/\text{min}$  provides a loop overflow of approximately twice the sample volume. Concentration was determined by quantitation against PTG standards. Microdialysis samples were further corrected using an *in vitro* probe calibration as described below.

### In vitro probe studies

Shunt microdialysis probes (BAS, MD-2100) with polyacrylonitrile membrane (MW cut-off 30,000 daltons) and window length of 25 mm were used in the *in vitro* experiments. The *in vitro* probe studies were all carried out at 37 °C using a thermostated heating block. The standard solution for *in vitro* recovery studies was a 1 mM solution of PTG in BSR pumped through the shunt with a BAS syringe pump (MD-1001) using various flow settings stated in the text. A shunt flow rate of 10  $\mu\text{L}/\text{min}$  was used for the *in vitro* probe calibration. BSR was perfused in a countercurrent fashion through the probe at 1  $\mu\text{L}/\text{min}$  and collected on-line to the HPLC injection system. The perfusate from the probe was directly connected to the injection needle in the injection valve. No additional tubing was added. Dialysate samples were collected and analyzed on-line every 6 minutes. Diverted bile samples from the shunt were connected to a refrigerated BAS Honeycomb fraction collector (BAS, MD-1200) using 20 cm of PE-10 tubing. The shunt samples were collected at set time points into pre-weighed vials and flow rates were determined gravimetrically assuming a density of 1 g/mL. The concentration of the shunt samples was determined by manual injection of samples under the same conditions used for dialysates. The percent extraction efficiency (by recovery experiment),  $EE_r$ , of the probe was calculated according to the following equation:

$$\%EE_r = \{[\text{dialysate}]/[\text{standard}]\} * 100$$

For in vitro studies requiring rapid changes in flow or concentration, the shunt was connected to as many as three syringe pumps using the BAS UniSwitch™ (BAS, MD-1508) (**F2**). The individual syringe pumps are allowed to stabilize and to operate continuously. An instantaneous change in flow rate or concentration was obtained using the liquid switch. The switch was changed manually at known time points to mimic changes in flow rate and analyte concentration in the bile.

### In vivo experiments

The probes used in the in vivo studies were custom shunt microdialysis probes with polyacrylonitrile membrane (MW cut-off 30,000 daltons) and window length of 30 mm. Since these probes had membrane lengths different than those used in the in vitro studies described above, they were calibrated individually in vitro before being placed in the animal. The in vitro calibration was validated by comparing the corrected dialysate concentrations to the actual concentrations in the bile in the diverted rat experiment. Probes

were calibrated with an in vitro recovery experiment at 37 °C using BSR at 1 μL/min as the perfusate and 1 mM PTG in BSR (standard) in the shunt at 10 μL/min. The probe setup, sample analysis and calculation of %EE<sub>r</sub> were as described in the in vitro section above. The in vitro calibration factor for the in vivo experiment was 72 ± 6%. One measurement was performed and errors were determined by propagation of error on multiple injections of standards (n > 6) and dialysates (n > 6).

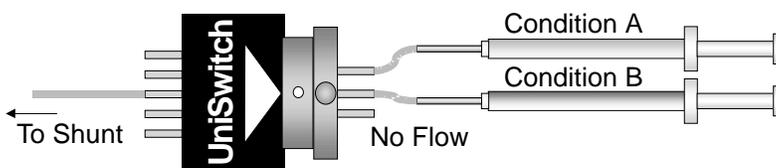
Rat surgery was performed in anesthetized animals according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Kansas and described in the shunt probe package insert (BAS, MD-2100). The probe was implanted in the anesthetized rats in the diverted fashion as shown in **F1**. Rats were lightly pre-anesthetized with halothane (Halocarbon Labs., River Edge, NJ) followed by intramuscular administration of 350 μL/kg Vetamine™ (100mg/mL ketamine hydrochloride, Mallinckrodt Vet., Mundelein, IL) with 10% Rompun™ (20mg/kg xylazine, Bayer,

Shawnee Mission, KS). Anesthesia was maintained by additional injections of 200 μL Vetamine™ approximately every 2 hours. The rat was placed on a heating pad kept at the low setting during the entire experiment. A narrow bore dosing cannula filled with heparinized Ringer's solution was ligated into the femoral vein. An intravenous dose of phenolphthalein (PT), 20 mg/kg in 40 μL DMSO was administered as described previously (4).

The in vivo probe was perfused with BSR at a flow rate of 1 μL/min with dialysate going directly to the injection loop of the analytical system. A 20-cm length of PE-10 tubing was used to connect the diverted, shunted bile to the BAS HoneyComb fraction collector. Dialysate flow rates were shown not to vary with PE-10 tubing lengths of 0-50 cm added to the downstream end of the shunt. This indicated that the additional PE-10 tubing does not increase the back pressure enough to cause ultrafiltration through the membrane to the dialysate. Twenty cm of PE-10 was the minimum length of tubing required for the shunt to reach the fraction collector. Samples were collected at predetermined time points into weighed, refrigerated, sealed vials. The bile and/or shunt flow rate was determined gravimetrically assuming a density of 1 g/mL. The PTG in bile was determined by manual injections of bile samples under the same analytical conditions as used for the dialysates.

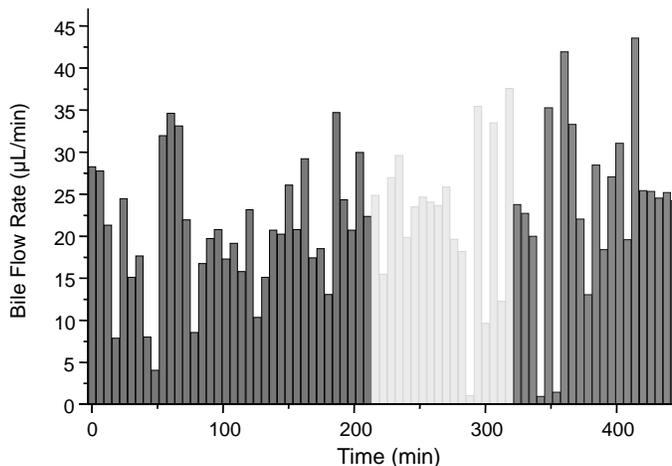
### F2

Illustration of the use of a liquid switch for in vitro experiments to mimic rapid changes in concentration or flow rate in the shunt of the microdialysis probe.



### F3

Bile flow rate in anesthetized rat with erratic bile flow. Timepoints 0-210 min (black) are baseline, 210-320 min (light grey) follow DMSO injection and 320-440 min (dark grey) follow injection of PT in DMSO.



## Results & Discussion

### In vivo experiments

**F3** shows the variation in bile flow in an anesthetized diverted rat experiment. The average bile flow was 22.1 ± 8.9 μL/min; however, the flow was erratic, varying over the range of 1.0 to 43.6 μL/min (n=75, 6-min time points). The reason for the erratic flow in **F3** is unknown. In an attempt to see if the flow rate variation was related to the dosing vehicle or to the PT dose, the bile

## T1

Average bile flow under different experimental conditions.

Experimental Condition	Average Bile Flow $\pm$ Standard Deviation ( $\mu\text{L}/\text{min}$ )	Number of Samples
baseline 0-3.6 h	21 $\pm$ 8	35
placebo 3.6-5.4 h	23 $\pm$ 9	19
PT dose 5.4-7.5	24 $\pm$ 10	21

## F4

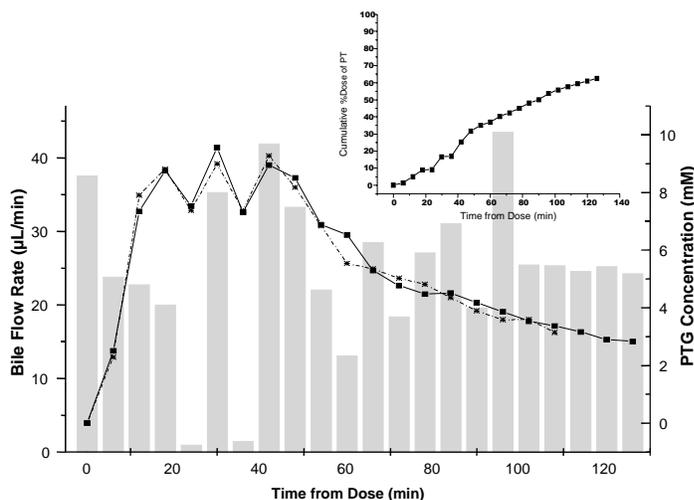
PTG and bile flow rate in anesthetized rat with erratic bile flow. Bile samples were collected every 6 minutes and 3 of 6  $\mu\text{L}$  of dialysate were analyzed on-line every 6 minutes.

■ = PTG concentration (mM) in dialysate corrected for EE (%EE<sub>r</sub>=72%).

\* = PTG concentration (mM) in bile.

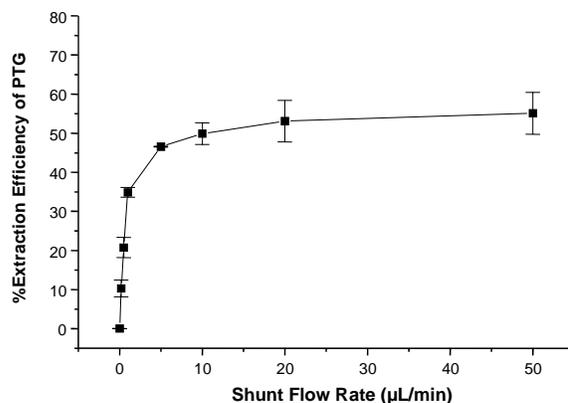
Grey bars = Bile flow rate ( $\mu\text{L}/\text{min}$ ).

Inset shows the total PT dose recovered from the bile with time.



## F5

% extraction efficiency (EE<sub>r</sub>) versus shunt flow rate. The shunt (1 mM PTG in BSR) flow was varied from 0.2 to 50  $\mu\text{L}/\text{min}$ . The perfusate (BSR) flow was constant at 1  $\mu\text{L}/\text{min}$ . Each measurement represents an average of  $n > 6$  dialysates  $\pm$  the standard deviation on the dialysates.



flow rate in the rat was monitored for 7.5 hours under the following conditions: 0 - 3.6 hours no injections (baseline), 3.6 - 5.4 hours rat given 50  $\mu\text{L}$  DMSO (placebo), and 5.4 - 7.5 hours rat given dose of PT in 50  $\mu\text{L}$  DMSO. Shading of the bars in **F3** shows the bile flow rate under these three conditions. **T1** gives the average bile flow and standard deviation for each group of samples.

Note that there appears to be no significant difference in the overall bile flow when the placebo and dose are compared to the baseline. This

indicates that the dose and vehicle are not responsible for the erratic flow. Other parameters such as the rat's temperature, the level of anesthesia, or the hydrostatic pressure in the bile duct may be the cause.

Despite the variation in flow rate, the profile obtained in **F4** shows that the comparison between dialysate and actual concentration measured in bile is quite good. An in vivo recovery value for PTG was calculated by taking the ratio of concentration in dialysate/bile for respective time points. The in vivo value of  $71 \pm 4\%$

(for time points 6-108 min) compared well with the in vitro calibration of  $72 \pm 6\%$ . In this experiment the total dose of PT was also determined (see inset, **F4**). After 126 min, 63% of the PT dose was recovered in the bile.

## In vitro experiments

Since it was observed in the in vivo experiments that the bile flow in the rat varies, the first set of in vitro experiments was to monitor the recovery of dialysates for shunt flow rates of 0.2-50  $\mu\text{L}/\text{min}$ . The EE<sub>r</sub> profile as a function of shunt flow is shown in **F5**. It is observed from the data that the recovery of analyte is independent of the shunt flow for flow rates  $> 5 \mu\text{L}/\text{min}$ . As the shunt represents a hydrodynamic environment, it is somewhat like a stirred pot where the limiting step for recovery is the diffusion of analyte through the probe membrane. This is in contrast to microdialysis in tissue, where the diffusion of analyte through the tissue is the rate dependent step. In these experiments at flow rates  $> 5 \mu\text{L}/\text{min}$  through the shunt, a steady state recovery is obtained. Due to this dynamic state in the shunt, an in vitro calibration of the shunt probe at 37 °C using a balanced concentration of bile salts across the membrane gives an accurate estimation of the in vivo concentration. A diverted bile experiment can be performed to validate the in vitro calibration (10).

In order to mimic erratic changes in bile flow, the liquid switch was set up to change between a syringe with 1 mM PTG at 20  $\mu\text{L}/\text{min}$  and no flow. The switch was changed back and forth every 10 minutes. Shunt samples were collected every 6 minutes. The predicted and measured shunt flow obtained from this on-off switching experiment is shown in **F6**. Notice that since the bile is collected in time increments that are different from the cycling time of the syringes, the predicted bile flow rates are averages that vary between 20 and 0  $\mu\text{L}/\text{min}$ . As seen in **F6**, the measured flow rate is in reasonable agreement with the predicted flow

### F6

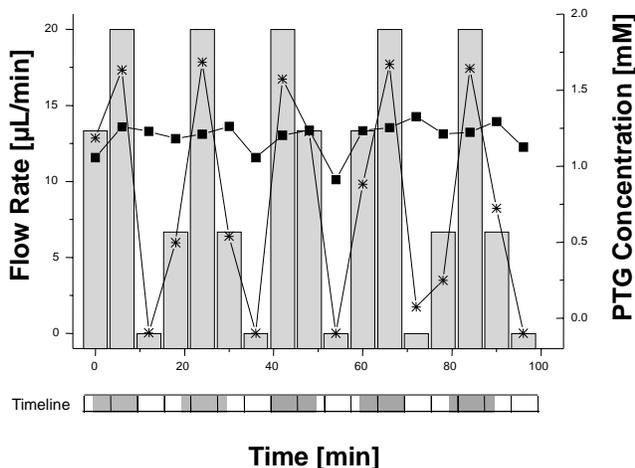
Tracking dialysate concentration under conditions of simulated on-off shunt flow.

■ = PTG concentration (mM) in dialysate corrected for EE (%EE<sub>r</sub>=53%).

\* = Measured flow rate from shunt.

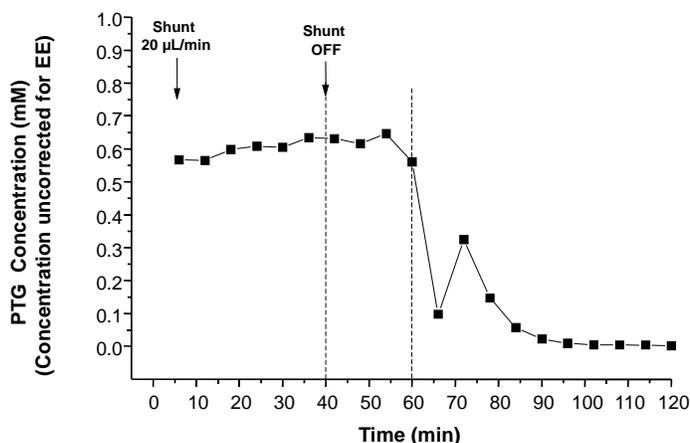
Grey bars = Predicted average flow rate in shunt.

Timeline:  
Dark gray represents the shunt flow on at 20 μL/min and white areas indicate that the flow is off.



### F7

Dialysate concentration monitored while flow in shunt is stagnant. The shunt flow of 20 μL/min (1 mM PTG in BSR) was turned off and the dialysates continued to be monitored. The perfusate was constant at 1 μL/min (BSR).



### F8

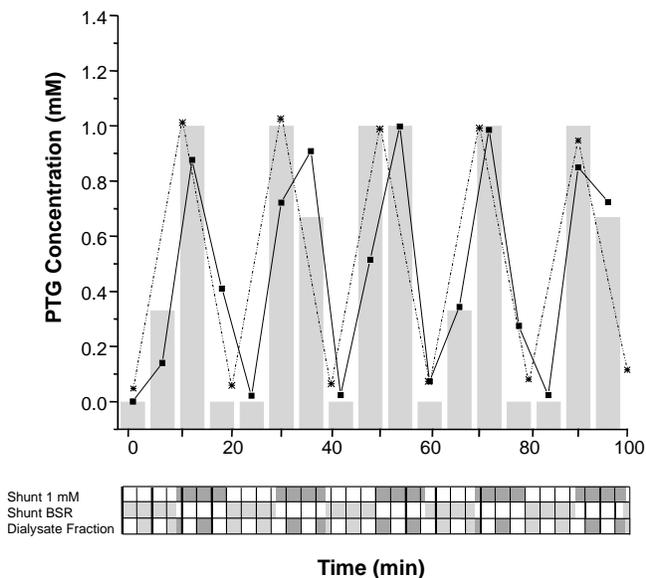
Tracking dialysate concentration under conditions of simulated changes in concentration in the shunt.

■ = PTG concentration (mM) in dialysate corrected for EE (%EE<sub>r</sub>=68%).

\* = PTG concentration (mM) in shunt.

Grey bars = Predicted PTG concentration (mM) in dialysate.

Timeline:  
Dark gray represents 1 mM PTG in the shunt and light grey represents BSR in the shunt. The white boxes in the dialysate fraction indicate the time interval where the sample is going to waste.



profile. This flow profile is not unlike the erratic bile flow measured in the in vivo experiment (F3). The dialysate concentration is consistent over the time frame of the experi-

ment, indicating that the recovery is constant and independent of the changes in shunt flow rate. The corrected dialysate concentration ( $1.2 \pm 0.1$  mM,  $n=16$ ) is in good agreement

with the actual concentration in the shunt of 1.0 mM PTG.

The concentration of analyte in the dialysate is expected to decrease if the solution in the shunt becomes completely stagnant. Under these conditions, where there is no movement of fluid in the shunt, it will take a finite amount of time for the concentration in the diffusion layer outside the membrane to become depleted. In order to get an estimate of how long this process would take in a shunt probe experiment, a probe was equilibrated with 1 mM PTG in the shunt at 20 μL/min and BSR (no PTG) in the perfusate at 1 μL/min. After 40 min of equilibration, the shunt flow was turned off but the perfusate flow was left on and the dialysate continued to be monitored. The results in F7 show that it takes approximately 15 minutes after the shunt flow is stopped to start to see a drop in dialysate concentration. This indicates that in the case of animal death or bile shutdown, the probe would continue to monitor the concentration that is sitting stagnant in the shunt for some time and it will not reflect the fact that the bile is not flowing. In an animal experiment, any animal movement (breathing, physical movement, vibrations, etc.) would cause stirring of the diffusion layer outside the membrane. Even slight stirring would serve to replenish the concentration outside the membrane and it would take much longer to observe a decrease in concentration.

When the concentration is changed in vitro, the probe responds very swiftly, profiling the concentration changes accurately. F8 illustrates an example where, using the liquid switch, the shunt concentration is varied between 1 mM PTG in BSR at 20 μL/min and BSR (no PTG) at 20 μL/min. The concentration is changed every 10 minutes. This profile was chosen to mimic extreme changes in concentration with constant flow rate in the shunt. The shunt flow was collected every 10 min and the dialysates were sampled for the last three minutes of

every 6-minute cycle. The predicted concentration in the shunt will vary between 1 and 0 mM. From **F8** it can be seen that the PTG concentration in the dialysates reasonably match the predicted concentration in the shunt, even under these conditions of extreme concentration changes.

### Conclusions

This study has shown that the shunt microdialysis probe is a valuable tool for profiling analytes in bile. The agreement in the in vivo experiment between the corrected dialysate concentration and the actual measured concentration in the bile was quite good. Although the reason for the variation in bile flow was not determined, these experiments show that the microdialysis shunt probe accurately tracks the

analyte concentration in the bile, independent of bile flow rate. The in vitro studies confirm that the probe reflects changes in analyte concentration even when the concentration or the bile flow rate changes rapidly.

### Acknowledgments

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