The Microdialysis Shunt Probe: The Influence of Bile Salts on Recovery of Caffeine and Phenolphthalein Glucuronide and Use of In Vitro Recovery Data for Calibration In Vivo

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Two contrasting analytes, caffeine and phenolphthalein glucuronide, were used in experiments with the BAS shunt microdialysis probe. This study shows that bile salts can affect the recovery of analytes through the microdialysis probe. Bile salts were shown to interact with phenolphthalein glucuronide but not with caffeine. Results also show that when bile salts are taken into account, in vitro calibration of the probe can be used to calculate the in vivo analyte concentration in bile. In vivo phenolphthalein glucuronide dialysate concentrations corrected with the in vitro recovery extraction efficiency (EE_r) were in good agreement with the in vivo concentrations measured in bile.

The development of a bile shunt microdialysis probe enables the study of the concentration of analytes in the bile in awake animals where the bile flow is intact (1-3). A diagram of the shunt microdialysis probe is shown in ***F1***. The probe's shunt, which carries the bile flow, is of appropriate dimensions for implantation into the bile duct of an adult rat. Suspended inside the shunt is a linear microdialysis probe that continuously samples low molecular weight compounds from the bile. The probe can be used in awake, freely moving animals for an extended period of time (2).

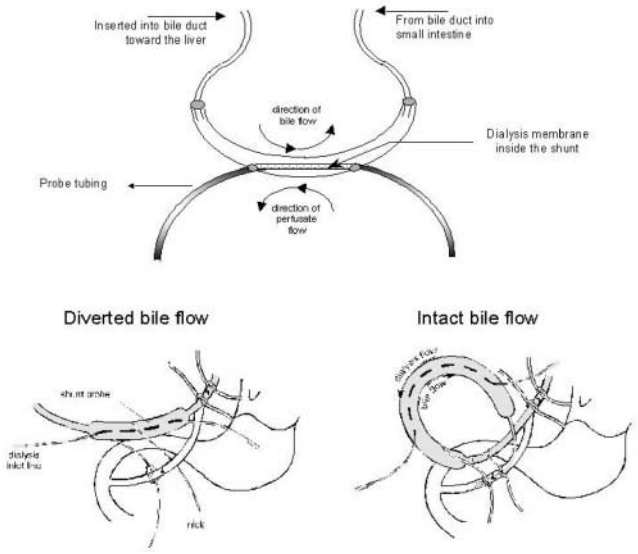
It is important to show that compounds of interest in the bile can be studied quantitatively using microdialysis. Compounds actively secreted in bile are usually polar molecules with molecular weights exceeding 300-400 g/mol. The actual molecular weight threshold for biliary excretion is species dependent, being 325 g/mol in the rat compared to 500 g/mol in man (4). Biotransformation of molecules in the liver to form adducts with glucuronic acid, glutathione, glycine or sulfate increases both the molecular weight and the polarity of the parent compound, making it more likely to find these conjugates in bile. Compounds secreted in bile are released to the gastro-intestinal lumen where they can undergo elimination, further metabolism or reabsorption into portal circulation. The process of reabsorption into the bile is referred to as enterohepatic circulation (EHC) and is important for compounds such as the bile acids, vitamins A, D_3 and B_{12}, folic acid, phenolphthalein, imipramine, indomethacin, antibiotics and estrogens (5).

The goals of this study were (1) to demonstrate that bile salts can affect the recovery of analytes through the microdialysis probe and (2) to show that, when bile salts are taken into account, calibration of the probe in vitro can be used to determine the in vivo concentration in the bile. In a previous study, it was shown that an imbalance of bile salts across the microdialysis membrane resulted in a net flow of water to the side of the membrane with the highest concentration of bile salts (6). The use of a 2% solution of bile salts in Ringer's (BSR) as the perfusate was sufficient to avoid loss in sample volume during in vivo dialysis experiments against bile.

In this study it is shown that the bile salts can have strong interactions with some analytes, thus affecting their ability to transport across the membrane. Two contrasting analytes, caffeine and phenolphthalein glucuronide, were chosen for comparison ***(F2)***. Caffeine (FW 194.2) is a small, water soluble compound

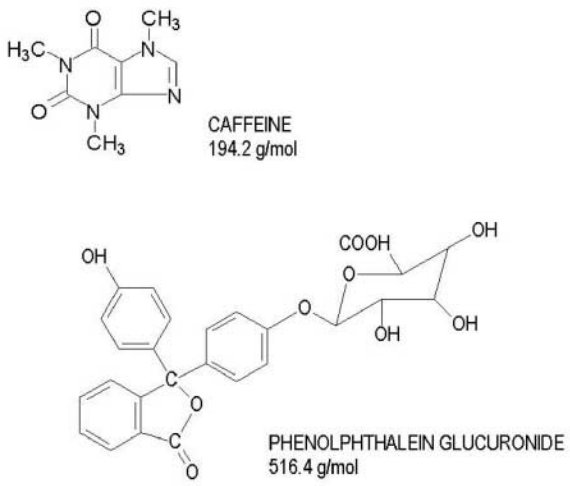
F1

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



F2

Structures of caffeine and phenolphthalein glucuronide.



which transports easily across typical microdialysis membranes. Caffeine has been used as an in vitro model compound for microdialysis probe calibration (7). It has also been characterized in vivo via microdialysis in various tissues (8-9) and used as an in vivo microdialysis internal standard (10). Recently, caffeine was used in an awake animal multiple-probe experiment in conjunction with activity monitoring (11).

Phenolphthalein glucuronide (FW 494.5) is an example of a classical glucuronide adduct known to undergo enterohepatic recycling (5,12). When phenolphthalein (PT) is administered, it is rapidly and extensively metabolized to phenolphthalein glucuronide (PTG) and secreted in the bile (12,13). Once in the gut lumen, PTG is hydrolyzed and PT is reabsorbed into the portal circulation (14). PT is typical of compounds that are biotransformed to high molecular weight adducts in the liver and then undergo enterohepatic cycling. The PT-PTG pair has been used as a model compound for the study of both liver function and EHC (15-18). In vivo profiles of phenophthalein glucuronide in anesthetized rats with diverted and intact bile flow have been obtained using microdialysis sampling (19).

In this study, the in vitro microdialysis transport properties of CA and PTG are compared with and without bile salts. The ability to monitor and quantitate PTG in vivo using microdialysis is demonstrated. The influence of the addition of bile salts on recovery data, and the use of in vitro recovery data to correct the in vivo microdialysis data, is shown to give good agreement with actual bile concentrations.

Materials and Methods

Reagents and Solutions

The following compounds were reagent grade or better and used as received from Sigma Chemical Co. (St. Louis, MO): caffeine (anhydrous grade), phenolphthalein, phenolphthalein glucuronic acid (sodium salt), bile salts (approximately 50% sodium cholate and 50% deoxycholate, Sigma #B-8756), KCl, $CaCl_2$, and NaCl. Ringer's solution was prepared from NaCl (155 mM), KCl (5.5 mM), and $CaCl_2$ (2.3 mM). Bile salt Ringer's solution (BSR) was prepared by dissolving 2 g of bile salts in 100 mL of Ringer's solution. HPLC grade acetonitrile and methanol, 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 (volume:volume) solvents and buffer followed by filtering through a 0.2-micron nylon filter under vacuum. The mobile phase was kept under helium pressure during use. All solutions were made using NANOpure (Barnstead Co., Boston, MA) deionized water.

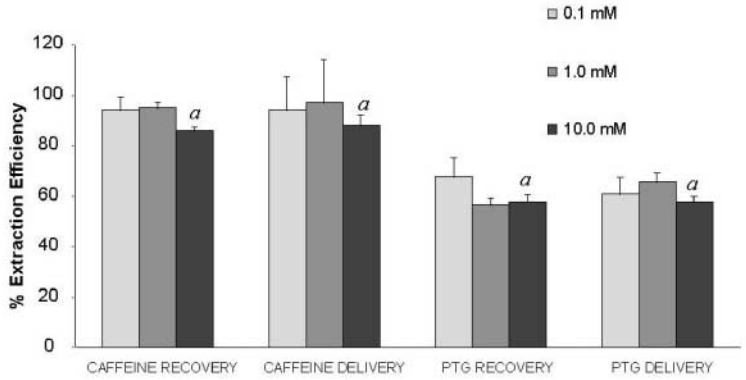
LC of Analytes

Standard solutions, dialysates, and bile samples were analyzed using a reverse phase liquid chromatography (LC) method. The LC

F3

Extraction efficiency of CA and PTG in the shunt probe at three concentration levels.

EE n∃8 dialysates and control samples ± absolute error by propagation of error on standards and dialysates. All solutions prepared in BSR. Data from a different probe designated by "a."



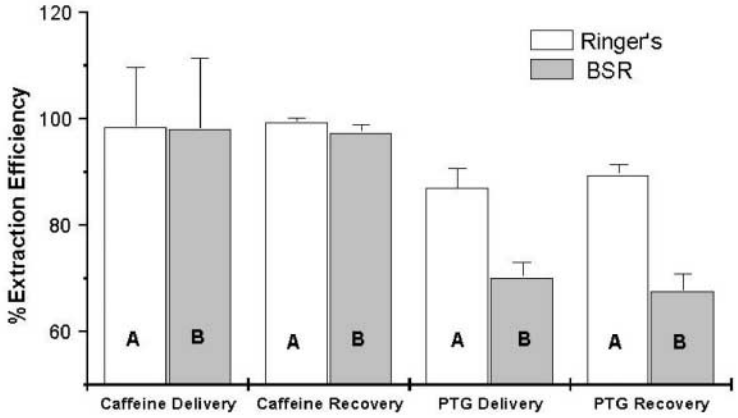
F4

Comparison of recovery and delivery of PTG and CA in Ringer's versus BSR

A) Measurements are averages (± average error) of three individual determinations over three days. Individual measurements consisted of n∃8 dialysates and controls with error propagated.

B) Bars indicate average from multiple measurements on the same day (n∃14 dialysates and controls) with error propagated.

All measurements performed with the same probe.



system consisted of a BAS 200 liquid chromatograph (Bioanalytical Systems, Inc. (BAS), West Lafayette, IN) with external UV absorbance detector (BAS MF-4660) operated at a detection wavelength of 230 nm. The column was a 3.2 x 100 mm, C_{18}, 3 µm particle size column (BAS MF-6213), preceded by a 3.2 x 15 mm, C_{18}, 7 µm guard column (BAS MF-6206) and a 0.5 µm in-line filter (BAS MF-8955). The mobile phase consisted of 20% methanol, 70.25% acetonitrile and 9.75% ammonium phosphate buffer (50 mM, pH=2.5). The mobile phase flow rate was 0.8 mL/min. The mobile phase and column were maintained at 35 °C. For in vitro experiments involving CA and PTG, a wavelength switching program was used to detect both analytes in the same analytical run (273 nm for CA, 230 nm for PTG). The approximate retention times for CA and PTG were 1.3 min and 3.5 min, respectively.

For the in vitro CA/PTG measurements, dialysates were analyzed on-line using a perfusion rate of 1 µL/min and an injection-loop fill time long enough to give at least 1.5 times the loop volume. For the in vivo PTG studies the injection system consisted of a Pollen-8 (BAS MD-1250) 10-port valve connected on-line and configured with a single inlet and two matched 6.5 µL injection loops. Dialysates were analyzed on-line by perfusion with BSR at a rate of 1 µL/min into the injection port for a 10-minute sampling interval. In this configuration, the two loops are alternately filled and then flushed to the LC column. The amount of dialysate analyzed is 6.5 µL out of every 10 µL. A loop overfill was used to insure accurate quantitation. Standards and bile samples were injected manually by overfilling the sample loop with a syringe.

In Vitro Extraction Efficiency

Custom (30 mm membrane) shunt microdialysis probes (BAS) were used in all experiments. The dialysis membrane was poly(acrylonitrile) with a molecular weight cutoff of 30,000 Daltons. The in vitro extraction efficiency was determined by placing the shunt probe into a 37° C water bath and connecting the inlet ends to syringe pumps (BAS MD-1001 and MD-1020) so that the flow through the shunt at 20 µL/min was counter-current to the perfusate flow at 1 µL/min. The analyte standard solution contained 1 mM each of CA and PTG made in either Ringer's solution or BSR. This solution was used in the shunt for recovery experiments (see below) and in the perfusate for delivery experiments. In each case, the analyte solution was dialyzed against a blank solution containing the same salts (i.e., Ringer's solution or BSR). The concentration of the dialysate was determined by LC and compared to injections of the 1 mM standard solution. The percent extraction efficiency (EE) in vitro was determined by both delivery (EE_d) and recovery (EE_r) experiments and was calculated as:

$$EE_d = \left(1 - \frac{C_{dialysate}}{C_{standard}}\right) \times 100$$

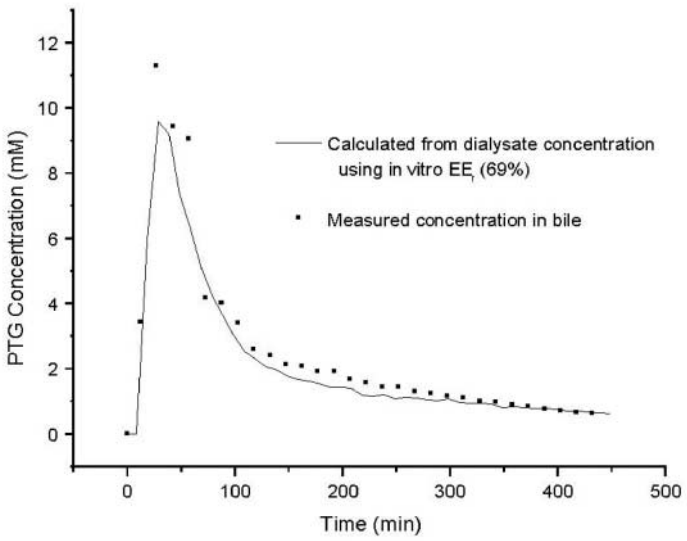
$$EE_r = \left(\frac{C_{dialysate}}{C_{standard}}\right) \times 100$$

In Vivo Studies

The shunt probe was implanted in anesthetized Sprague-Dawley rats according to the general surgical procedures outlined in the standard shunt probe package insert (BAS

F5

Comparision of PTG concentration measured in bile and calculated from dialysates using in vitro probe calibration.



MD-2100) and following protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Kansas. In order to calibrate the probe in vivo, the bile flow was diverted and only the end of the shunt proximal to the liver was inserted into the bile duct ***(F1)***. This allowed the bile to flow across the membrane and into a fraction collector for direct analysis of the bile. Bile from the shunt was collected in capped vials at 15-minute intervals into a refrigerated fraction collector held at 4 °C (BAS HoneyComb, MD-1201). Samples were injected manually. The shunt probe membrane was perfused with BSR at a rate of 1 µL/min and the dialysates were analyzed on-line.

After the analysis of several predose samples to insure no interferences, an i.v. dose of 19 mg/kg PT was administered via the femoral vein. The PT was dissolved in 20 µL of DMSO and carefully layered in a 50 µL syringe (BAS MF-5003) as follows: 10 µL DMSO, the 20 µL dose, and 10 µL DMSO. The dose was given over about 1 min via a 6 cm PE-10 femoral cannula that was then flushed with 300 µL of Ringer's solution over a period of about 2 min. This dosing protocol was developed to deliver the entire dose, to minimize the amount of DMSO and to avoid precipitation of the PT in the cannula.

Results and Discussion

In Vitro Experiments

The EE for a given probe and set of operating parameters depends on the nature of the analytes and the sample matrix. In a previous study, it was shown that in order for EE_d to equal EE_r, the bile salt concentration in the perfusate must approximate the concentration in the shunt. Dialyzing bile without bile salts in the perfusate resulted in a net flow of water through the membrane into the shunt. This gave rise to a decrease in sample dialysate volume collected (6). The use of BSR as the perfusate was adequate to balance the bile salts and to avoid artifacts created by the osmotic flux of water across the membrane.

To ensure that the EE of neither PTG nor caffeine was concentration dependent, EE_d and EE_r values were determined for the shunt probe over the range 0.1 to 10 mM in each analyte. The results in ***F3*** show that the EEs were consistent, and the EE_r matched the EE_d for both analytes. The EE values were independent of concentration over the range 0.1-10 mM. Other researchers have also shown that probe EE is independent of concentration. For example, EE values measured for caffeine were shown to have no dependence on concentration over the range spanning 25 to 300 µM (7).

The in vitro EE of PTG and CA in Ringer's solution and in BSR are shown in ***F4***. The CA extraction efficiencies determined by delivery and by recovery are the same in both Ringer's solution and in BSR (overall average 99 ± 11%). The diffusion of caffeine across the dialysis membrane is independent of the presence of bile salts. This contrasts with the results observed for PTG. The EE of PTG is less than caffeine, which would be anticipated simply due to its larger molecular weight. However, it is observed that the average EE (by delivery and recovery) for PTG in Ringer's solution is 88 ± 4% while in BSR it is 69 ± 3%. This decrease in the ability of PTG to diffuse across the dialysis membrane in the presence of bile salts indicates that this compound, with a polar (sugar) moiety and a non-polar (phenolphthalein) moiety, interacts with the bile salt micelles. This interaction was also reported by Vonk, et al., who observed the association of PTG with biliary micelles in sedimentation pattern studies (20). In BSR, PTG extraction efficiencies by delivery and by recovery are equal, as expected. Although the EE in BSR is lower than in Ringer's solution, the value is reproducible and easily quantitated by the analytical method used in this study.

In Vivo Experiments

In ***F5*** the in vivo concentration vs. time profile for PTG in diverted bile samples is compared with the bile dialysates after correction using the in vitro EE_r of 69 ± 3%. The calculated and measured values are in good agreement along the entire profile. An in vivo EE by recovery ($EE_{(r,\ in\ vivo)}$) can be calculated by the following equation:

$$EE_{(r,\ in\ vivo)} = \left(\frac{C_{dialysate}}{C_{bile}} \right) \times 100$$

using $C_{dialysate}$ and C_{bile} for time-corresponding samples. $EE_{(r,\ in\ vivo)}$ for

the samples from 27 through 417 minutes averaged 61 ± 6%. The in vivo EE (61 ± 6%) is within experimental error of the in vitro EE (69 ± 3%).

Although in vitro EE_r values are not always considered applicable for in vivo microdialysis, flow of bile in the shunt should produce a hydrodynamic situation such that diffusion through the membrane is the limiting factor, and the in vitro extraction efficiencies may thus be applied to in vivo data. This contrasts with microdialysis sampling in tissue, where the recovery of analytes may be dependent on the nature and tortuosity of the tissue (21). In this experiment, the bile flow rate averaged 10.1 ± 2.3 µL/min (n = 32 samples, 15 minutes each). As the shunt probe resembles sampling from a stirred pool, it is reasonable that the in vitro calibration of the probe at 37 °C using analyte solutions prepared in BSR and shunt flow rates > 20 µL/min would apply to in vivo measurements.

At the peak concentration, the calculated PTG level in the dialysate is lower than in the corresponding bile sample. This may be due to the fact that all the bile is collected in 15 minute sample intervals and analyzed. However, the configuration of the on-line LC system results in loss of part of the dialysate (6.7 µL injected out of 10 µL) during each interval. Consequently, if the concentration change at the peak is rapid (since only a fraction of the dialysate is analyzed), the on-line analysis may have missed a portion of the analyte at its peak concentration.

Conclusions

In a microdialysis shunt probe, the extraction efficiency of CA was greater than PTG, independent of concentration, and not affected by bile salts. PTG interacts with bile salts and its EE is less in the presence of bile salts than in Ringer's solution. When the composition of bile salts in the perfusion solution was matched to the shunt matrix in vitro (i.e., BSR to BSR), the EE_d for PTG was the same as EE_r. The in vitro EE for PTG in BSR showed no concentration dependence in the range 0.1 - 10 mM, was reproducible and therefore suitable for use in analyzing data from in vivo experiments. Furthermore, using the in vitro EE_r to correct bile dialysate PTG concentrations gave good agreement with bile sample levels measured directly.

Acknowledgments

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References

1. D.O. Scott and C.E. Lunte, Pharm. Res. 10 (1993) 335.
2. C. Gunaratna, S.M. Lunte and H. Zuo, Current Seps. 13:3 (1994) 80.
3. M.E. Hadwiger, M. Telting-Diaz and C.E. Lunte, J. Chrom. B 655 (1994) 235.
4. M.R. Dobrinska, J. Clin. Pharmacol. 29 (1989) 577.
5. C.D. Klaassen and J.B. Watkins III, Pharmaco. Rev. 36:1 (1984) 1.
6. K.E. Heppert, W.H. Flora and M.I. Davies, Current Seps. 17:2 (1998) 61.
7. Y. Zhao, X. Liang and C.E. Lunte, Anal. Chim. Acta 316 (1995) 403.
8. L. Stahle, S. Segersvard and U. Ungerstedt, Life Sci. 49 (1991) 1853.
9. L. Stahle, P. Arner and U. Ungerstedt, Life Sci. 49 (1991) 1835.
10. C.I. Larsson, Life Sci. 49 (1991) PL-73.
11. K.E. Heppert and M.I. Davies, Current Seps. 18:1 (1999) 3.
12. P. Millburn, R.L. Smith and R. Williams, Biochem. J. 105 (1967) 1275.
13. W.A. Colburn, P.C. Hirom, R.J. Parker and P. Milburn, Drug Metab. Disp. 7 (1979) 100.
14. R.J. Parker, P.C. Hirom and P. Millburn, Xenobiotica 10 (1980) 689.
15. M.F. Kanz, R.F. Whitehead, A.E. Ferguson, L. Kaphalia and M.T. Moslen, J. Pharm. Meth. 27 (1992) 7.
16. L.G. Rochelle and L.R. Curtis, Toxicology 86 (1994) 123.
17. P. Johnson and P.A. Rising, Xenobiotica 8 (1978) 27.
18. F.L.S. Tse, F. Ballard and J.M. Jaffe, J. Pharm. Meth. 7 (1982) 139.
19. K.E. Heppert and M.I. Davies, Anal. Chim. Acta 379 (1998) 359.
20. R.J. Vonk, P. Jekel and D.K. Meijer, Naunyn-Schmiedeberg's Arch. Pharmacol. 290 (1975) 375.
21. P.M. Bungay, P.F. Morrison and R.L. Dedrick, Life Sci. 46 (1989) 105.