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Improving microdialysis extraction efficiency of lipophilic eicosanoids

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Abstract

Microdialysis recovery of the lipophilic analytes prostaglandin B_2, leukotriene B_4 and C_4 was studied in vitro. Relative recovery (RR) through different commercially-available microdialysis probes for prostaglandin B_2 and leukotrienes was examined using different flow rates. The enhancing effect at different concentrations of binding agents such as α, β, γ-cyclodextrins (α, β, γ-CD) on the microdialysis RR for different eicosanoids was evaluated. Small organic molecules such as ethanol, propylene glycol and dimethyl sulfoxide (DMSO) were studied in terms of their effect on enhancing RR. Inclusion of arachidonic acid in either the perfusion fluid or the sample medium caused the microdialysis RR for these hydrophobic analytes to be increased.
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1. Introduction

Microdialysis sampling is a well-established technique that allows collection of macromolecule-free samples from complex biological matrices [1,2]. In vivo microdialysis sampling has been used extensively for collection of hydrophilic analytes such as neurotransmitters from rodent brain. Although in vivo microdialysis sampling has become routine practice for sampling hydrophilic analytes, there have been many difficulties reported in the literature with respect to microdialysis sampling of important lipophilic analytes from complex biological matrices [3–5].

There are several different analytical challenges associated with in vivo microdialysis sampling schemes for lipophilic analytes. The first difficulty is that lipophilic analytes, particularly drugs, often exhibit high protein binding. High protein binding causes a small fraction of the overall analyte concentration to be in the unbound state within the sample space. Depending on the analyte of interest and its in vivo concentration, there may be significant analytical challenges associated with making a concentration measurement [6]. Low analyte concentrations coupled with microdialysis relative recoveries (RR) that are often below 100%

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can cause severe limitations with detection limits for many lipophilic analytes. A second difficulty is that lipophilic analytes often adhere to plastic or glass surfaces used during analysis. This second problem is significant during microdialysis sampling because of the polymeric nature of the microdialysis membrane and the plastic and/or glass (fused silica) outlet tubing and sample collection vials that are inherent to microdialysis sampling techniques.

There have been different approaches described for enhancing RR of lipophilic analytes. Ståhle and Carneheim described approaches for improving oleic acid RR in vitro using albumin [3]. By including albumin in the perfusate, the RR of oleic acid was nearly doubled as compared with controls. Others have included albumin in the perfusate to prevent non-specific adsorption of different analytes [7]. The difficulty with albumin addition to the microdialysis perfusion fluid is that it adds back to the microdialysate what microdialysis sampling was originally intended to prevent, principally large macromolecules. With protein already in the solution, the only analytical method that could be used for analyte quantitation without further sample preparation would be immunoassay methods, which are tolerant to the protein in the sample. It should be noted that a vast majority of microdialysis samples are analyzed using liquid chromatographic techniques.

Several different approaches for enhancing microdialysis RR that are amenable for HPLC detection have been reported in the literature. These methods include lipo-microdialysis [8], cyclodextrin (CD) enhancement [9], and solid-support enhancement [10]. Enhancement approaches for microdialysis sampling that allow injection onto an HPLC without substantial sample clean up would be more universal with respect to the analysis of hydrophobic analytes from microdialysis samples.

Leukotrienes, prostaglandins and thromboxanes are members of the important class of lipid mediators known as eicosanoids and are metabolites of the major cell membrane constituent arachidonic acid (AA). Eicosanoids play important mediating roles during inflammatory and allergic reactions [11,12]. There has been a significant amount of interest in the physiological and pathophysiological roles of eicosanoids [13,14]. Off-line and on-line microdialysis sampling of eicosanoids has been reported [15,16]. CDs have been included in both the perfusion fluid and sample medium during microdialysis sampling of eicosanoids from mammalian cell culture [16].

RR is generally defined as shown in Eq. (1), where $C_{dialysate}$ and C_{sample} denote the analyte concentration in the dialysate and external sample medium, respectively. In this paper, we systematically explore the use of different reagents that can be added to the microdialysis perfusion fluid for improving microdialysis sampling of lipophilic eicosanoids.

$$RR = \frac{C_{dialysate}}{C_{sample}} \tag{1}$$

Although enhancement agents such as CD have been previously described to improve eicosanoid microdialysis sampling, the agent was also added to the sample solution, which causes more complexity in the mass transport analysis and is not a viable solution for in vivo microdialysis applications. Part of the analyses described in this work involves calculating a mass transfer coefficient for different analytes across different membrane materials. The rationale for choosing to calculate a mass transfer coefficient rather than reporting RR for each membrane is that RR is highly dependent upon the surface area of the membrane material. The mass transfer coefficient is considered to be an independent property of the membrane and would be expected to be a constant and independent of analyte concentration, perfusion fluid flow rate and membrane surface area. The equation for calculating an effective mass transfer coefficient is shown in Eq. (2), where $C_{dialysate}$ is the dialysate concentration; C_{sample}, the sample concentration;

$$\frac{C_{dialysate}}{C_{sample}} = 1 - \exp(-KA/Q) \tag{2}$$

K, the mass transfer coefficient; A, the membrane area and Q is the volumetric flow rate [17].

2. Experimental

2.1. Chemicals

AA, prostaglandin B_2 (PGB_2), leukotriene B_4 (LTB_4), leukotriene C_4 (LTC_4), leuokotriene D_4 (LTD_4), and leukotriene E_4 (LTE_4) were purchased from Cayman Chemical (Ann Arbor, MI, USA). α-CD and DL-dithiothreitol were obtained from Sigma (St. Louis, MO, USA). β- and γ-CD were donated by Wacker Chemical Corporation (Adrian, MI, USA). All other chemicals were reagent grade or better.

2.2. Equipment

RP-HPLC was performed using a Synergi 4μ Max-RP 80A (250 mm × 2.00 mm) column (Phenomenex, Torrance, CA, USA). The HPLC mobile phase consisted of acetonitrile − 0.1 M sodium phosphate (45:55, v/v%), pH 2.6. HPLC data was acquired using a Thermoseparations Products (San Jose, CA, USA) P1000 pump with a UV 1000 detector controlled by PC1000 software. The mobile phase flow rate was 0.2 ml/min and the injection volume was 5 μl. The UV detector was set to 278 nm for optimal detection of PGB_2 and leukotrienes.

Microdialysis was performed using a 1 ml Hamilton non-metallic syringe (Hamilton Company, Reno, NV, USA) with a CMA-102 syringe pump (CMA/Microdialysis, North Chelmsford, MA, USA) and microdialysis probes purchased from either Bioanalytical Systems (BAS-4, West Lafayette, IN, USA) or CMA Microdialysis (CMA-20, North Chelmsford, MA, USA). Probes with membrane lengths of either 4 or 10 mm were used. Table 1 shows the properties for these dialysis membranes.

2.3. Preparation of standards and samples

PGB_2 stock solutions were prepared by dissolving solid PGB_2 in ethanol to a final concentration of 500 μg/ml and stored at − 80 °C. Prior to experimentation, a standard solution of PGB_2 was prepared in 0.9 w/v% NaCl. The standard solution of PGB_2 was stable for a week when stored in the refrigerator in a glass vial. LTB_4, LTC_4, and AA were supplied as 100 μg/ml standards in ethanol and stored at − 80 °C.

2.4. Microdialysis experiments

The perfusion fluid flow rates used for this study were between 0.7 and 3.5 μl/min. The collected sample volume was 15 μl. Experiments were performed in both quiescent and well-stirred solutions at room temperature (22.5–23.5 °C). Analytes were spiked into 1.5 ml vials containing 0.9% w/v NaCl. Eicosanoid sample concentration was determined prior to experimentation by taking two separate sample aliquots and subjecting these to HPLC analysis. To ensure that sample concentrations remained constant throughout the microdialysis process, two additional sample aliquots were removed after collection of dialysate samples from each set of two consecutive flow rate trials (twice during the experiment) and after completion of the experiment. Four dialysate samples were collected at each flow rate and analyzed for eicosanoid content and the last three samples were averaged together to calculate RR.

3. Results and discussion

3.1. Analyte stability

Others have reported decreasing microdialysis RR values, particularly for hydrophobic analytes, when flow rates were reduced from 1.0 to 0.5 μl/min [16]. This is in contrast to what is generally expected during microdialysis sampling and defined by Eq. (2). There are a few possibilities that explain such behavior during microdialysis sampling. If the flow rate was started at 0.5 μl/min and then raised to 1.0 μl/min, it is possible that non-specific adsorption sites in the tubing material have not been saturated since the 1.0 μl/min perfusion fluid flow rate would give a higher mass flow rate rather than concentration flow rate. An alternate explanation would be instability of the analyte. Since lower flow rates require longer collection times to reach a target volume

Table 1
Microdialysis probe physical properties

	PC	PAN	CUP	PES
Commercial supplier	CMA	BAS	CMA	CMA
Length (mm)	4 or 10	4 or 10	3	4
Outer radius (μm)	250	170	120	250
Inner radius (μm)	200	120	95	200
Wall thickness (μm)	50	50	25	50
Molecular weight cutoff	20 000	29 000	6000	100 000

The data provided here is that given by the manufacturers of the microdialysis probes. It is not known if the radii are for dry or wet membranes.

for analysis, it is possible that the analyte was unstable during collection.

Because of concerns with respect to these previously published observations with hydrophobic analytes having a decreased recovery when the perfusion flow rate is decreased during microdialysis sampling, we ensured that our samples were stable throughout the microdialysis sampling process. PGB_2 concentrations in the sample medium were monitored during the microdialysis sampling process and the relative standard deviation (R.S.D.) was determined. The R.S.D. of PGB_2 concentration in the medium during microdialysis sampling was determined for four separate experiments. In these experiments seven aliquots of the external medium were obtained (two before microdialysis sampling, three during microdialysis sampling, and two after completion of the experiment). The R.S.D. of the concentrations tested ranged between 5.2 and 9.7% (5.2, 6.1, 6.1, 9.7) during these experiments. This indicates that PGB_2 was relatively stable at the concentrations used during the microdialysis sampling process. Note that the μM concentrations tested here significantly higher than what would be expected in vivo for these analytes. The R.S.D. of LTB_4 concentration during LTB_4 experiments was between 3.6 and 10.0% (9.8, 3.6, 7.3 and 10.0).

While analyte stability problems were not noted for PGB_2 and LTB_4 at the concentrations used in these experiments, they were observed for LTC_4, LTD_4 and LTE_4. LTC_4, LTD_4 and LTE_4 are unstable compounds and are required to be stored at -80 °C. The analytical signal for standards of these analytes decreased rapidly when placed in a plastic vial at room temperature and protected from light. In order to stabilize these analytes, an antioxidant was tried. Addition of 1 mM dithiothreitol (DTT) to the standard solution was effective in preventing LTC_4 decomposition for 5–6 h. However, for LTD_4 and LTE_4, 1 mM DTT only provided stable concentrations for 2 h. In addition, the degradation kinetics were not as rapid when analytes were sampled from glass rather than plastic vials. This suggests that some of the problems with the stability of these analytes may be attributed to either partitioning or adsorption processes with the plastic.

3.2. *Mass transfer coefficients*

To be able to compare and contrast the mass transport properties of the different microdialysis membranes we chose to calculate a mass transfer coefficient for each membrane for the analytes PGB_2 and LTB_4. Assuming that diffusion coefficients are constant through the membrane and independent of perfusion fluid flow rate, K, the mass transfer coefficient, should be a constant.

Tables 2 and 3 show the calculated mass transfer coefficient values for PGB_2 and LTB_4 for different membrane materials at different flow rates. The data exhibited in these tables show that the mass transfer coefficient was generally independent of microdialysis perfusion fluid flow rate. In these studies, the flow rates were tested in a random manner to avoid correlation of RR and/or mass transfer coefficients with direction of the flow rate change.

Table 2
Experimentally determined mass transfer coefficients for PGB_2 through different microdialysis membranes

Manufacturer, material and MWCO	Area (mm^2)	C_{sample} (μM)	Q_d (μl/min)	RR (%)	K (mm/min)	Average K
CMA/20	6.28	39	2.5	5.9 ± 0.2	$(2.4 \pm 0.1) \times 10^{-2}$	$(1.8 \pm 0.6) \times 10^{-2}$
PES (4 mm)			2.0	5.2 ± 1.7	$(1.7 \pm 0.6) \times 10^{-2}$	
Polyethersulfone (100 kDa)			1.5	7.3 ± 0.7	$(1.8 \pm 0.2) \times 10^{-2}$	
			1.0	8.2 ± 0.3	$(1.4 \pm 0.1) \times 10^{-2}$	
CMA/11	2.26	39	2.5	3.9 ± 0.3	$(4.4 \pm 0.3) \times 10^{-2}$	$(4.3 \pm 0.5) \times 10^{-2}$
CUP (3 mm)			2.0	5.1 ± 0.2	$(4.6 \pm 0.2) \times 10^{-2}$	
Cuprophan (6 kDa)			1.5	6.9 ± 0.3	$(4.8 \pm 0.2) \times 10^{-2}$	
			1.0	8.7 ± 0.4	$(4.0 \pm 0.2) \times 10^{-2}$	
			0.7	11.4 ± 0.2	$(3.7 \pm 0.1) \times 10^{-2}$	
BAS-BR	4.02	60	2.5	6.8 ± 0.2	$(4.4 \pm 0.2) \times 10^{-2}$	$(4.0 \pm 0.5) \times 10^{-2}$
PAN (4 mm)			2.0	8.0 ± 0.7	$(4.2 \pm 0.4) \times 10^{-2}$	
Polyacrylonitrile (29 kDa)			1.5	10.0 ± 0.4	$(3.9 \pm 0.2) \times 10^{-2}$	
			1.0	13.4 ± 0.6	$(3.6 \pm 0.2) \times 10^{-2}$	
BAS-BR	4.02	39	2.5	7.7 ± 0.5	$(5.0 \pm 0.3) \times 10^{-2}$	$(4.8 \pm 0.7) \times 10^{-2}$ [a]
PAN (4 mm)			2.0	10.2 ± 0.6	$(5.3 \pm 0.3) \times 10^{-2}$	
Polyacrylonitrile (29 kDa)			1.5	13.5 ± 0.7	$(5.4 \pm 0.3) \times 10^{-2}$	
			1.0	17.5 ± 0.9	$(4.8 \pm 0.3) \times 10^{-2}$	
			0.7	18.2 ± 1.6	$(3.5 \pm 0.3) \times 10^{-2}$	
BAS-LN	10.05	30	2.5	17.2 ± 0.9	$(4.7 \pm 0.3) \times 10^{-2}$	$(3.7 \pm 0.6) \times 10^{-2}$ [a]
PAN (10 mm)			2	20.3 ± 0.7	$(4.5 \pm 0.2) \times 10^{-2}$	
Polyacrylonitrile (29 kDa)			1.5	26.7 ± 2.2	$(4.7 \pm 0.5) \times 10^{-2}$	
			1	28.3 ± 0.5	$(3.3 \pm 0.1) \times 10^{-2}$	
			0.7	19.2 ± 2.7	$(1.5 \pm 0.2) \times 10^{-2}$	
CMA/20	15.71	27	2.5	24.2 ± 1.2	$(4.4 \pm 0.3) \times 10^{-2}$	$(3.5 \pm 0.9) \times 10^{-2}$
PC (10 mm)			2	24.8 ± 1.4	$(3.6 \pm 0.2) \times 10^{-2}$	
Polycarbonate (20 kDa)			1.5	28.0 ± 1.5	$(3.1 \pm 0.2) \times 10^{-2}$	
			1	31.4 ± 5.1	$(2.4 \pm 0.5) \times 10^{-2}$	
			0.7	55.9 ± 6.5	$(3.7 \pm 0.7) \times 10^{-2}$	
CMA/20	6.28	24	2.5	9.6 ± 3.1	$(4.0 \pm 1.4) \times 10^{-2}$	$(3.4 \pm 1.4) \times 10^{-2}$
PC (4 mm)			2	8.2 ± 0.4	$(2.7 \pm 0.1) \times 10^{-2}$	
Polycarbonate (20 kDa)			1.5	8.4 ± 0.2	$(2.1 \pm 0.1) \times 10^{-2}$	
			1	24.7 ± 1.6	$(4.5 \pm 0.4) \times 10^{-2}$	
			0.7	26.2 ± 0.9	$(3.4 \pm 0.1) \times 10^{-2}$	

Data were obtained by microdialysis sampling of PGB_2 from 1.5 ml PGB_2/saline plastic sample vial and HPLC-UV analysis of analyte. Microdialysis was performed under quiescent conditions at room temperature. Results are expressed as mean ± S.D. ($n = 3$).
[a] Indicates significantly different at the 95% confidence level for ANOVA.

For PGB_2, cuprophan (CUP) and polyacrylonitrile (PAN) membranes had the greatest overall mass transfer coefficients among the different microdialysis membranes tested. It should be noted that the polyethersulfone (PES) membrane had the lowest mass transfer coefficient. Yet, the PES membrane had a larger surface area than either CUP and PAN and a significantly larger MWCO of 100 kDa as compared with the 29 and 6 kDa MWCO for PAN and CUP membranes, respectively. This is in contrast to what was experimentally observed for LTB_4 across the different membrane materials. For LTB_4, the CUP membrane exhibited the highest overall mass transfer coefficient, yet this was much higher than observed for PAN, polycarbonate (PC) and PES membranes.

The observation that CUP membrane has the highest calculated mass transfer coefficient has been noted in the literature by others for different analytes [18]. Mass transfer differences among different membrane materials for similar analytes have also been previously noted [19,20]. Although CUP and PAN membranes tend to exhibit high

Table 3
Experimentally determined mass transfer coefficients for LTB_4 through different microdialysis membranes

Membrane (length)	Area (mm^2)	C_{sample} (μM)	Q_d (μl/min)	RR (%)	K (mm/min)	K average
PES (4 mm)	6.28	10	3.0	5.4 ± 0.3	$(2.7 \pm 0.2) \times 10^{-2}$	$(2.3 \pm 0.5) \times 10^{-2}$
			2.0	7.3 ± 0.6	$(2.4 \pm 0.2) \times 10^{-2}$	
			1.0	10.6 ± 2.2	$(1.8 \pm 0.4) \times 10^{-2}$	
CUP (4 mm)	3.01	10	3.5	3.9 ± 0.7	$(4.7 \pm 0.9) \times 10^{-2}$	$(3.7 \pm 0.9) \times 10^{-2a}$
			2.5	4.9 ± 0.1	$(4.2 \pm 0.1) \times 10^{-2}$	
			0.7	9.4 ± 0.7	$(2.3 \pm 0.2) \times 10^{-2}$	
PAN (4 mm)	4.02	5	3.5	2.4 ± 0.2	$(2.2 \pm 0.1) \times 10^{-2}$	$(1.8 \pm 0.3) \times 10^{-2a}$
			2.0	4.2 ± 0.3	$(2.1 \pm 0.2) \times 10^{-2}$	
			1.5	5.1 ± 0.5	$(1.4 \pm 0.1) \times 10^{-2}$	
			1.0	5.3 ± 0.3	$(1.4 \pm 0.1) \times 10^{-2}$	
			0.7	7.7 ± 0.3	$(2.0 \pm 0.2) \times 10^{-2}$	
PAN (4 mm)	4.02	10	3.0	2.6 ± 0.5	$(1.9 \pm 0.4) \times 10^{-2}$	$(1.5 \pm 0.4) \times 10^{-2}$
			2.0	2.7 ± 0.4	$(1.3 \pm 0.2) \times 10^{-2}$	
			1.5	4.1 ± 0.3	$(1.6 \pm 0.1) \times 10^{-2}$	
			0.7	7.4 ± 0.8	$(1.3 \pm 0.1) \times 10^{-2}$	
PAN (10 mm)	10.05	10	2.5	3.4 ± 0.1	$(0.9 \pm 0.1) \times 10^{-2}$	$(0.9 \pm 0.2) \times 10^{-2a}$
			1.5	5.9 ± 1.0	$(0.9 \pm 0.2) \times 10^{-2}$	
			0.7	11.2 ± 0.6	$(0.8 \pm 0.1) \times 10^{-2}$	
PC (4 mm)	6.28	10	3.5	2.3 ± 0.8	$(1.3 \pm 0.4) \times 10^{-2}$	$(2.6 \pm 0.7) \times 10^{-2a}$
			2.5	5.6 ± 0.9	$(2.3 \pm 0.4) \times 10^{-2}$	
			1.5	13.2 ± 1.4	$(3.4 \pm 0.4) \times 10^{-2}$	
			0.7	27.3 ± 0.7	$(3.6 \pm 0.1) \times 10^{-2}$	
PC (10 mm)	15.71	10	2.5	14.4 ± 2.2	$(2.5 \pm 0.4) \times 10^{-2}$	$(2.4 \pm 0.5) \times 10^{-2a}$
			1.5	21.1 ± 1.6	$(2.3 \pm 0.2) \times 10^{-2}$	
			0.7	42.4 ± 1.7	$(2.5 \pm 0.1) \times 10^{-2}$	

Data were obtained by microdialysis sampling of LTB_4 from 1.5 ml LTB_4/saline plastic sample vial and HPLC-UV analysis of analyte. Microdialysis was performed under quiescent conditions at room temperature. Results are expressed as mean ± S.D. (n = 3).
[a] Indicates significantly different at the 95% confidence level for ANOVA.

mass transfer coefficients, they often have lower microdialysis RR values as compared with PES and PC membranes because of their much lower surface area due to their smaller external radius. The PC membrane exhibited medium values for its mass transfer coefficient values for PGB_2 and LTB_4. However, the large surface area of the PC membrane compensates for its medium mass transfer coefficient making it ideal for obtaining high microdialysis RR for these hydrophobic analytes.

It is interesting to note that despite the similarity in molecular weight between PGB_2 (334.5) and LTB_4 (336.5) the mass transfer coefficients through the different microdialysis membranes were quite different. In particular, PGB_2 exhibited higher mass transfer coefficients than LTB_4. This may be explained by the solubility differences between the two since PGB_2 and LTB_4 have manufacturer reported solubility values of 2 versus 1 mg/ml in H_2O, respectively.

In these studies, PGB_2 and LTB_4 were able to cross the microdialysis membrane materials tested in this study despite being hydrophobic. Although the octanol–water partition coefficients for PGB_2 and LTB_4 have not been reported in the literature, these analytes are products of AA metabolism and their log P values would be expected to be similar to the value for AA which has a reported log P of 6.98 [21]. During microdialysis sampling it would not be surprising if lipophilic analytes such as AA partitioned into the polymeric membrane as has been previously hypothesized by others for lipophilic alcohols [20]. Furthermore, Mary et al. reported difficulties in obtaining recovery through both PC (20 kDa) and PES (100 kDa) membranes

(purchased from CMA/Microdialysis) with trimethylpsoralen that has a log P of 3.14, whereas other psoralens with log P values of approximately 2.00 did not cause problems during microdialysis sampling with these membranes [22]. This is not unreasonable since different dialysis membrane materials are characterized as hydrophilic versus hydrophobic.

CUP membrane is generally considered hydrophilic, whereas PES and PAN are considered hydrophobic [23,24]. Despite these broad classifications, CUP membrane gave the highest mass transfer coefficient for hydrophobic analytes. Since the outlet tubing and other materials such as collection vials used in the study were the same, except for the PAN 10 mm membrane which has different inlet and outlet tubing associated with it, the differences observed in mass transfer coefficients can be attributed to differences in the ability of the hydrophobic analyte to diffuse across the dialysis membrane. Microdialysis membranes that are commercially available only represent a small fraction of the different hemodialysis membranes that are used in clinical practice [24]. Furthermore, caution is warranted when describing these different materials. Many different dialysis membranes are different blends of different polymeric monomers. Thus calling a membrane ‘‘polycarbonate’’ or ‘‘polyacrylonitrile’’ does not fully describe the true chemical nature and ultimately the hydrophobicity of such a membrane. Unfortunately, the microdialysis membranes that are commercially available are proprietary and thus getting information about the blend percentages is not possible.

The mass transfer coefficients for PGB_2 and LTB_4 through the different membranes were similar as determined by ANOVA between the different flow rates. For PGB_2 statistical differences were noted between the different flow rates used for two different PAN membranes. However, for the three separate experiments on PAN membranes, the value of the determined mass transfer coefficient was similar. LTB_4 seemed to be more problematic with respect to differences in the calculated mass transfer coefficient. These differences are most likely associated with it being more hydrophobic than PGB_2. Despite some of the membranes having statistical differences between the calculated mass transfer coefficient at different flow rates, the expected trend of having RR increase as the perfusion fluid flow rate is reduced was always observed.

PAN membranes exhibited high mass transfer coefficients and are available as metal-free microdialysis probes. Because this work is part of a larger project that requires metal-free probes, most of the characterization studies described here were performed using PAN membranes. Fig. 1 shows the concentration dependence for the microdialysis RR of PGB_2 and LTB_4 in a quiescent solution across a PAN membrane. However, when the medium is well stirred the concentration dependence diminishes (data not shown). A possible reason for this concentration dependence of RR during unstirred experiments may be the adsorption of the analyte onto the membrane. When lower concentrations are used, a larger analyte fraction is likely to be captured on the membrane.

3.3. Cyclodextrin perfusion fluid additives

Because of their low endogenous concentrations in physiological fluids (nM), the analysis of eicosanoids or other hydrophobic analytes can be quite challenging if RR through a microdialysis probe is low or if significant adsorption occurs. One method for increasing the overall sample concentration during microdialysis sampling is to include different agents in the perfusion fluid to enhance the mass transport of analyte across the membrane. Passive diffusion can be facilitated if coupled with a chemical reaction, such as a complex formation reaction, on the inside of the membrane. In this study, CDs were chosen as the complex agent because they are non-selective, and thus bind with a variety of different analytes. An additional advantage for using CDs as enhancing agents for samples that are quantified using LC methods is that acetonitrile and methanol compete for the CD cavity. Although no literature values appear to be available for the binding between different CDs and the analytes studied in this report, the literature reported formation binding constant between β-CD and prostaglandin E_2 (not used in this study, but similar in structure to PGB_2) is $10^{3.27}$ or 1862 per M [25].

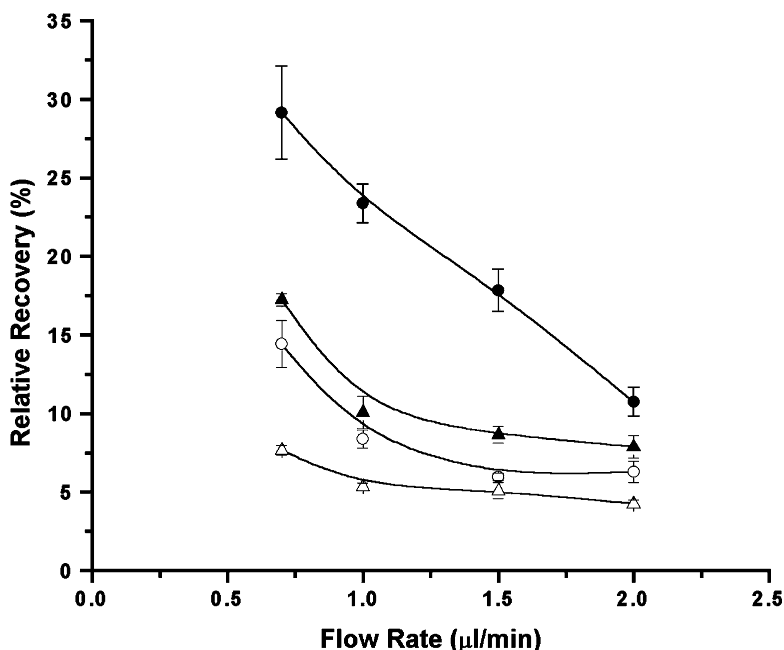

Fig. 1. Concentration dependence of RR in quiescent medium. A 4 mm BAS-BR probe was used for all experiments. (▲) 5 μM PGB_2, no additive in perfusion fluid; (●) 10 μM PGB_2, no additive in perfusion fluid; (△) 5 μM LTB_4, no additive in perfusion fluid; (○) 8 μM LTB_4, no additive in perfusion fluid. Error bars represent mean ± S.D. for n = 3 samples collected at each flow rate. Regression lines are not meant to be fits of the data.

Table 4 shows the RR enhancement for PGB_2 across a PAN 4-mm probe when different concentrations of α-, β- and γ-CD were included in the perfusion fluid. In general, γ-CD did not significantly enhance RR for PGB_2. Inclusion of α- and β-CD in the perfusion fluid significantly enhanced RR. However, in two separate experiments, the inclusion of γ-CD caused a statistical decrease in the RR between the control and γ-CD-containing perfusion fluid. In some cases for high flow rates, inclusion of γ-CD in the perfusion fluid enhanced the RR by a few percent. Although these changes are statistically significant as determined by a *t*-test at the 95% confidence level, it is likely that between-day variation is being exhibited in these specific situations. Furthermore, few researchers have used CD as perfusion fluid modifiers during microdialysis sampling so the true expected trends when applying this method are not fully understood at the present time.

The range of PGB_2 RR enhancement for the different types and concentrations of CD added ranged between no significant enhancement and enhancements of about 60% greater than control RR. No specific trends of increased RR enhancement as a function of flow rate decrease were observed among the different flow rates used. This was in contrast to previous work that we have published where RR enhancement was directly correlated with flow rate [9,26,27]. These observed differences may be due to these analytes being more hydrophobic than previous analytes we have studied to demonstrate CD enhancements of RR during microdialysis sampling. The combination of all these results suggests that for PGB_2 inclusion of CD in the microdialysis perfusion fluid does not provide significant benefit.

Table 5 shows the RR enhancement for LTB_4 across a PAN 4-mm probe when different concentrations of α-, β- and γ-CD were included in the perfusion fluid. When CDs were included in the perfusion fluid, LTB_4 RR was significantly more enhanced than PGB_2. These differences between the analytes may be due to either the solubility

Table 4
CD enhancement for PGB_2 across a PAN membrane

Sample	Flow rate (μl/min)	RR% (control)	[CD]	RR after adding CD (%)	Enhancing effect (%)
PGB_2 (10 μM)	0.7	29.2±3.0	5.14 mM α-CD	35.1±1.9	20.4
	1.0	23.4±1.2	5.14 mM α-CD	25.6±0.1	9.5
	1.5	17.8±1.4	5.14 mM α-CD	20.5±0.9	14.9
	2.0	10.7±0.9	5.14 mM α-CD	15.0±0.1[c]	39.2
	0.7		4.41 mM β-CD	36.5±0.8	25.3
	1.0		4.41 mM β-CD	27.9±2.2	19.3
	1.5		4.41 mM β-CD	24.1±4.2	–[a]
	2.0		4.41 mM β-CD	16.5±0.1[c]	53.8
	0.7		3.86 mM γ-CD	29.8±5.0	–[a]
	1.0		3.86 mM γ-CD	15.9±2.6	−31.9[b]
	1.5		3.86 mM γ-CD	13.8±1.1	−21.7[b]
	2.0		3.86 mM γ-CD	10.3±0.6	–[a]
	0.7		19.3 mM γ-CD	32.7±1.2	–[a]
	1.0		19.3 mM γ-CD	21.0±0.9	–[a]
	1.5		19.3 mM γ-CD	15.6±0.9	–[a]
	2.0		19.3 mM γ-CD	12.7±0.3	18.4
	0.7		38.6 mM γ-CD	30.0±0.3	–[a]
	1.0		38.6 mM γ-CD	23.5±0.6	–[a]
	1.5		38.6 mM γ-CD	16.7±0.6	–[a]
	2.0		38.6 mM γ-CD	13.1±0.6	21.6
PGB_2 (5 μM)	0.7	17.0±1.7	5 mM α-CD	26.7±1.7[c]	57.4
	1.0	13.5±0.8	5 mM α-CD	19.2±0.9[c]	41.8
	1.5	9.4±0.6	5 mM α-CD	12.9±0.4[c]	36.7
	2.0	6.3±1.0	5 mM α-CD	10.1±0.8[c]	59.6
	0.7		5 mM β-CD	23.1±0.7[c]	35.9
	1.0		5 mM β-CD	16.0±0.5[c]	18.5
	1.5		5 mM β-CD	10.3±0.8[c]	–[a]
	2.0		5 mM β-CD	8.1±0.7[c]	27.9
	0.7		5 mM γ-CD	17.8±0.6	–[a]
	1.0		5 mM γ-CD	13.5±1.2	–[a]
	1.5		5 mM γ-CD	9.2±0.6	–[a]
	2.0		5 mM γ-CD	6.7±0.7	–[a]

Experiments were performed in a quiescent medium. Results are expressed as mean±S.D. ($n = 3$).
[a] Indicates no significant (95% confidence) RR increase between control and CD-containing perfusion media as determined by a t-test.
[b] Indicates a statistical (95% confidence) RR difference (decline) between control and CD-containing perfusion media as determined by a t-test.
[c] Indicates a statistical difference (95% confidence) between RR enhancements obtained with α-CD vs. β-CD containing perfusion media at the same flow rate as determined by a t-test.

differences, thermodynamic binding constant differences, or kinetic on/off rate differences with the CD. For LTB_4, γ-CD did enhance RR at the lower flow rates, but not at higher perfusion fluid rates.

For some in vitro applications, the addition of CD to the medium rather than perfusion fluid to prevent non-specific adsorption has been described during microdialysis sampling [16]. Fig. 2 shows the RR differences for LTB_4 when 5 mM β-CD was added to the microdialysis perfusion fluid and when the same β-CD concentration was added to both the perfusion fluid and the sample medium. Having β-CD in the perfusion fluid or in both the perfusion fluid and external sample medium greatly enhanced LTB_4 RR. There are significant differences between when β-CD was included only in the perfusion fluid versus β-CD inclusion in both the perfusion fluid and sample medium. In

Table 5
CD RR enhancement for LTB_4 in a quiescent medium for a PAN membrane

Sample	Flow rate (µl/min)	RR% (control)	[CD]	RR% after adding CD	Enhancement effect (%)
LTB_4 (5 µM)	0.7	7.7±0.3	13.3 mM α-CD	19.2±1.2	151.2
	1.0	5.3±0.3	13.3 mM α-CD	13.8±0.9	158.2
	1.5	5.1±0.5	13.3 mM α-CD	7.3±0.8	44.1
	2.0	4.2±0.3	13.3 mM α-CD	5.1±1.1	–[a]
	0.7		5 mM α-CD	16.8±0.3[b]	119.1
	1.0		5 mM α-CD	13.8±0.4[b]	157.5
	1.5		5 mM α-CD	12.2±1.2	140.8
	2.0		5 mM α-CD	9.4±0.6	121.0
	0.7		5 mM β-CD	21.9±1.3[b]	185.9
	1.0		5 mM β-CD	16.4±0.2[b]	207.5
	1.5		5 mM β-CD	11.5±0.3	125.6
	2.0		5 mM β-CD	8.4±0.7	98.4
	0.7		5 mM γ-CD	14.5±0.8	88.9
	1.0		5 mM γ-CD	12.8±1.8	138.8
	1.5		5 mM γ-CD	6.2±0.6	–[a]
	2.0		5 mM γ-CD	5.3±0.7	–[a]

Experiments were performed in a quiescent medium. Results are expressed as mean±S.D. (n = 3).

[a] Indicates no significant (95% confidence) RR increase between control and CD-containing perfusion media as determined by a *t*-test.

[b] Indicates a statistical difference (95% confidence) between RR enhancements obtained with α-CD vs. β-CD containing perfusion media at the same flow rate as determined by a *t*-test.

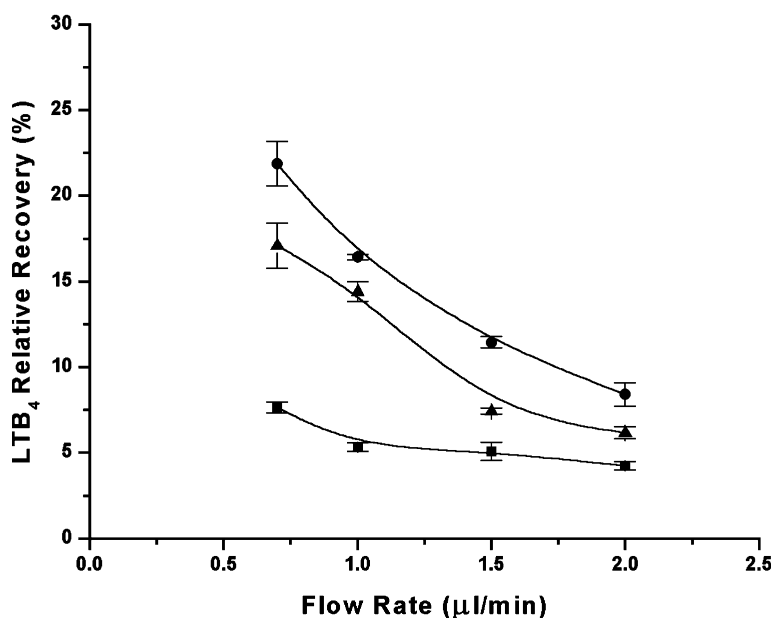

Fig. 2. Influence of β-CD on LTB_4 RR. Microdialysis sampling was performed using a BAS-BR 4 mm probe in 5 µM LTB_4 quiescent solution. (■) Control; (●) 5 mM β-CD in the perfusion fluid and (▲) 5 mM β-CD in the perfusion fluid and external to the probe. Error bars represent mean±S.D. for n = 3 samples collected at each flow rate. Regression lines are not meant to be fits of the data.

the case with the β-CD in the sample medium and perfusion fluid, the RR was lower than with β-CD in only the perfusion fluid. This may be explained by the reduction in the free fraction of LTB_4 in the sample medium when β-CD is included in the sample medium. Furthermore, a LTB_4/CD complex with a much higher molecular weight than free LTB_4 would be expected to cause a decrease in RR.

We have previously observed significant enhancement differences when including CD in the perfusion fluid between microdialysis membranes that have different internal and external diameter [9]. Since such low enhancements were observed with the CD as compared with our previous experience with this approach, we determined whether or not much greater enhancements could be obtained using a different membrane material. Our past experience has suggested that for CD enhancement, PC membranes with an internal diameter of 400 μm perform significantly better than PAN and CUP membranes which have smaller internal diameters (Table 1). Table 6 shows the RR enhancement for LTB_4 through a 4-mm PC membrane using different flow rates with 1% β-CD included in the perfusion fluid. The RR enhancements observed are significantly greater than using the PC membrane as compared with PAN and are dependent on flow rate.

3.4. RR enhancements with small molecules

Observable differences in RR occurred with different concentrations of either LTB_4 or PGB_2 in the external sample medium. Because of the analyte hydrophobicity, we determined the potential usefulness of including small organic molecules rather than CD in the microdialysis perfusion fluid. These small organic molecules included: AA, ethanol, dimethyl sulfoxide and propylene glycol. The choice of these analytes was based on their use in many biological sample preparations.

Prostaglandins and leukotrienes are products of AA metabolism. The effect of adding AA to the external sample medium or the microdialysis perfusion fluid to enhance RR was studied for PGB_2 across a PAN probe in a well-stirred sample medium. The results were compared with RR of that obtained in well-stirred sample with and without β-CD added into the perfusion fluid. Addition of 0.328 mM AA to the perfusion fluid gave a similar enhancing effect as addition of 5 mM β-CD for PGB_2 (Fig. 3). Fig. 3 also shows that when AA is included in the sample medium, there was a significant enhancing effect to PGB_2 RR as compared with control. These results may be explained by the similar structure and physical properties of AA to that of PGB_2. These results indicate that consideration of other chemicals in the external sample medium milieu may significantly affect microdialysis RR.

In addition to AA, ethanol, DMSO and propylene glycol were tested for their enhancing effects. These results are shown in Table 7. DMSO appeared to increase the RR of PGB_2 as well as AA. It is unknown what the long term biological effects would be by infusing a 1% DMSO solution through a localized area. Propylene glycol is metabolized to lactic acid. It did enhance the RR for PGB_2, but not as much as DMSO and AA. This may be because a lower concentration was used since 7 μg/ml (0.092 mM) is reported to be the safe upper toxicological limit.

Although inclusion of these small molecules showed to be useful for in vitro microdialysis sampling, their effectiveness in vivo would cer-

Table 6
β-CD enhancement for LTB_4 across a 4-mm PC membrane

Flow rate (μl/min)	Control (RR%)	1% (8.82 mM) β-CD in perfusate (RR%)	Enhancement (%)
3.5	2.3 ± 0.8	12.8 ± 0.9	460
2.5	5.6 ± 0.9	18.4 ± 0.7	229
1.5	13.2 ± 1.4	26.3 ± 1.4	98.8
0.7	27.3 ± 0.7	46.2 ± 0.3	69.0

Experiments were performed in a quiescent medium. Results are expressed as mean ± S.D. (n = 3).

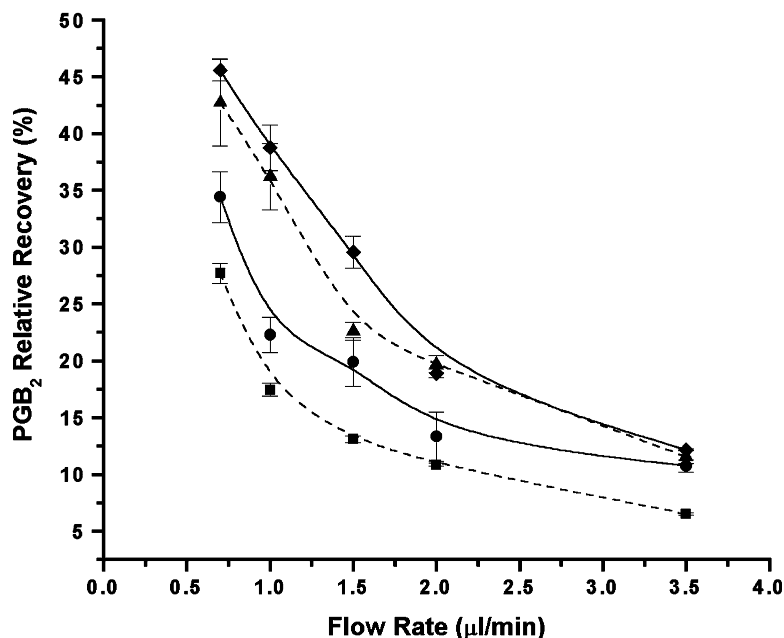

Fig. 3. BAS BR-4 probe in well stirred medium with a 5.0 μM PGB_2 solution, (■), Control; (●), 0.328 mM arachidonic acid (AA) included in sample medium; (▲), 0.328 mM AA included in perfusion fluid; (◆), 5 mM β-CD included in perfusion fluid. Error bars represent mean ± S.D. for n = 3 samples collected at each flow rate. Regression lines are not meant to be fits of the data.

tainly have to be evaluated and validated prior to use. A significant advantage of microdialysis sampling is that small amounts of mass are actually exchanged between the sample and perfusion fluid. However, whether or not these small molecules would significantly alter localized physiology would have to be determined.

4. Conclusions

Microdialysis sampling of hydrophobic analytes was tested using a variety of different membranes with various agents included in the perfusion fluid. Significant differences between PGB_2 and LTB_4 were found with respect to their mass transfer coefficients across commercially-available membranes. These differences are most likely attributable to both the analyte hydrophobicity and the membrane hydrophobicity.

Several different agents including CD, AA, ethanol and DMSO were tried as recovery enhancers for the eicosanoids. The use of CD or AA seemed to provide the most significant increases in RR. However, these increases are no more than two or three times greater than the RR obtained under control conditions.

Table 7
Enhancing effect of small molecules included in perfusion fluid

Sample	Microdialysis flow rate (μl/min)	RR of control exp (%)	Additives in perfusion fluid	RR after adding additives (%)	Enhancing effect (%)
PGB_2 (5 μM)	0.7	27.7 ± 0.9	2 v/v% Ethanol	30.6 ± 1.5	10.4
			1 v/v% DMSO	42.2 ± 3.6	52.6
			0.092 mM Propylene glycol	34.1 ± 0.2	23.0
			0.33 mM AA	42.7 ± 3.9	54.9

A PAN membrane (BAS-4) was used. The samples were stirred. Results are expressed as mean ± S.D. (n = 3).

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