Journal of Chromatography, 578 (1992) 157–163
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6392

In vivo sampling using loop microdialysis probes coupled to a liquid chromatograph

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(Received March 6th, 1992)

ABSTRACT

Microdialysis probes with longer membranes (20–100 mm) provide increased relative recovery over traditional shorter probes (1–4 mm) developed for neuroscience applications. The characterization and optimization of "straight through" or "loop type" probes for use in subcutaneous tissue are considered. Membrane area, probe size, inlet and outlet tubing dimensions, and flow-rate are examined for their effects on relative recovery, the total collection rate, and bulk flow through the membrane wall. Polyacrylonitrile and regenerated cellulose membrane fibers with different geometries were compared. Sampling probes used fibers 3–10 cm long. Inlet and outlet tubing was varied from 25 to 110 μm I.D. with lengths of 10 to 50 cm. Probe configurations optimized for relative recovery, flow-rate, and utility for *in vivo* use are presented. Utilizing microdialysis probes with large membrane surface areas results in relative concentration recovery of greater than 50% at flow-rates of greater than 5 μl/min. Therapeutic drug monitoring in subcutaneous tissue of awake animals is explored.

INTRODUCTION

The monitoring of low-molecular mass substances in biological systems is important. Microdialysis probes have been shown to be a practical method for *in vivo* sampling due to their small size, ability to continuously monitor awake animals, and ease of automation. Microdialysis probes have been used to study small molecules in the brain, blood, and subcutaneous tissue among others [1–15]. Mathematical models for relative recovery and interstitial concentration have been previously presented [1,2,15–17]. In almost all of these studies small concentric-type microdialysis probes with membrane lengths of less than 10 mm were used. Probe length is an important consideration. Several models use assumptions that are not valid for longer membranes (10–50 mm). It has, for example, been assumed that the concentration on the inside of the dialysis membrane remains constant during measurements [1]. In microdialysis probes the concentration of analyte in the perfusate increases along the membrane fiber from the inlet to outlet ends. This transport phenomenon leads to relative recoveries (extraction fraction) that reach a constant over a range of flow-rates for membranes several centimeters in length.

Traditional microdialysis probes have several limitations. Flow-rates are typically less than 5 μl/min. The inlet and outlet tubing must be large enough in diameter so that substantial back-pressure is not created, while remaining small enough to avoid significant concentration dispersion. Variations in perfusate flow-rate can create imprecision, especially at low flow-rates (<1 μl/min). Relative concentration recoveries are often

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0378-4347/92/$05.00

low (5–30%), with the small volumes collected placing large demands on analysis methodology.

Longer membrane microdialysis probes provide a variety of operating options. The constant high recoveries of loop probes allow for increased perfusion rates and more control over the desired perfusion rate. Although loop probes with longer membranes have increased recoveries, the problem of back-pressure due to the small dimensions of outlet tubing becomes more problematic. In addition, such probes will not be very useful for *in vivo* monitoring of blood vessels or brain regions due to size constraints. Nevertheless, loop microdialysis probes are ideal for use in subcutaneous tissue, the buccal cavity, and muscle tissue.

EXPERIMENTAL

Microdialysis probes

Microdialysis probes were constructed using fused-silica tubing from Polymicro Technologies (Phoenix, AZ, USA) with internal diameters of 25, 50, 75, 100, 181, and 258 μm and outer diameters of 151, 187, 143 190, 343, and 354 μm, respectively. The inlet tubing was 5 cm while outlet tubing was variable from 10 to 50 cm long. Two membrane polymers were used: polyacrylonitrile (PAN) with a molecular mass cut-off (MWCO) of 30 000 daltons, 210 μm I.D. and 320 μm O.D. and regenerated cellulose (RC) (Spectrum Medical Industries, Los Angeles, CA, USA) with MWCO of 6000 and 9000 daltons, 150 μm I.D. and 170 μm O.D. To reinforce the membranes, a 75-μm stainless-steel wire (Fort Wayne Metals, Fort Wayne, IN, USA) was threaded through the lumen of the membrane. Unmodified membranes were glued to the fused silica using epoxy cement. The structure of these microdialysis probes is illustrated in Fig. 1.

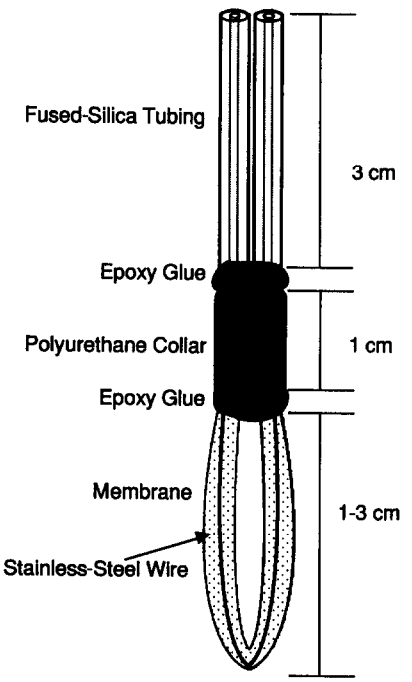

Fig. 1. Typical loop-type microdialysis probe used for all experiments. Materials are described in text.

Microdialysis

All microdialysis experiments utilized a CMA 100 microinjection pump from Bioanalytical Systems (West Lafayette, IN, USA) equipped with a 1-ml Hamilton (Reno, NV, USA) gas-tight syringe. For *in vitro* studies the sample was continuously stirred with a magnetic stirring plate maintained at 27 ± 1°C. Samples were collected at 5-min intervals, and flow-rates were determined gravimetrically.

Chromatography

Chromatography was carried out on a BAS 200A liquid chromatograph from Bioanalytical Systems. For all separations a Biophase C_{18} (BAS), 3 μm, 100 × 3.2 mm I.D. column maintained at 35°C was used with a 5-μl sample loop. Detection was accomplished with the UV detector set at 250 nm and 0.002 a.u.f.s. For the determination of acetaminophen, a mobile phase of 100 m*M* phosphate buffer (pH 6.1) with 5% acetonitrile and a flow-rate of 1 ml/min was used.

Reagents

Acetaminophen was purchased from Sigma (St. Louis, MO, USA). All buffers and perfusates were made with reagent-grade materials. All solutions were made with double-distilled, deionized water and filtered through 0.45-μm nylon fil-

ters. Acetonitrile was HPLC grade purchased from Baxter (Muskegon, MI, USA).

In vivo microdialysis

A 350-g rat was anesthetized using 0.1 ml/100 g ketamine–xylazine. A 4-cm PAN probe was implanted into the subcutaneous tissue over the shoulder. The animal was allowed to recover for 18 h. The animal was placed in an awake animal bowl (BAS/CMA) and the probe was perfused at a rate of 3 μl/min for 1 h. A dual swivel system was used to prevent twisting of the inlet and outlet tubes. The outlet tubing (110 μm I.D. Teflon) was limited to 30 cm to reduce back-pressure. After 1 h the animal was injected intraperitoneally with 0.9 ml of a 10 mg/ml solution of acetaminophen. Samples were collected every 10 min for 2.5 h. Samples were frozen prior to analysis by chromatography.

RESULTS AND DISCUSSION

The first variables explored were the outlet tubing length and internal diameter. In microdialysis the membrane fiber is permeable in both directions. If the outlet tubing produces significant back-pressure the perfusate can be pushed out through the membrane by ultrafiltration. Using a 5.4-cm PAN probe at a flow-rate of 4.9 μl/min the above-mentioned parameters were examined. When outlet tubing length was increased and diameter decreased there was a significant drop in outlet flow-rate. A true dialysis probe functions only by passive diffusion across the membrane. Any bulk flow of liquid across the membrane in either direction will complicate the interpretation of sampled concentrations.

Fig. 2 illustrates the situation for four different diameter tubes and lengths. The use of 75 or 50 μm I.D. outlet capillary for a probe of this size would cause significant problems even at lengths of 10 cm. The disadvantage of the back-pressure in the outlet tubing is traded against the advantage of decreased internal volume. The same experiments were repeated for the RC membrane. The results were similar, but not as severe. Due to the much smaller permeability (MWCO, 9000 *versus* 30 000), the RC probe was able to withstand more back-pressure.

The next parameter examined was membrane length. Using a flow-rate of 4.9 μl/min and 100 μm I.D. outlet tubing, probes were made with PAN membrane lengths of 3.0, 5.3, 6.9, and 9.5 cm. The outlet flow-rate dependence is illustrated in Fig. 3. The increased membrane and outlet tubing length significantly increased the ultrafil-

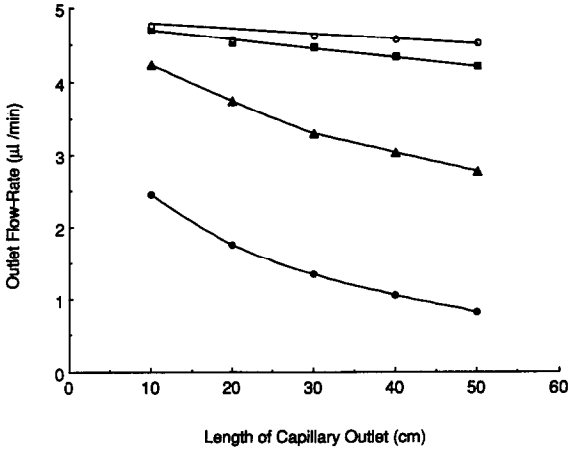

Fig. 2. Effect of different outlet tubing diameters and lengths on outlet flow-rate (○, 110 μm; ■, 100 μm; ▲, 75 μm; ●, 50 μm). A 5.4-cm PAN membrane was used with a 4.9 μl/min inlet flow-rate.

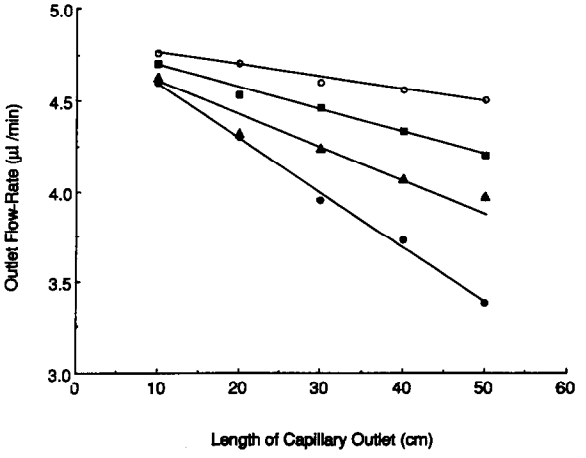

Fig. 3. Effect of membrane length and outlet length on outlet flow-rate for PAN membranes (○, 3.0 cm; ■, 5.7 cm; ▲, 6.9 cm; ●, 9.5 cm). A constant inlet flow-rate of 4.9 μl/min was used.

tration leakage. Although the leakage increased, the relative recovery for each probe was surprisingly constant. In a similar experiment, RC probes of 9.0, 5.5, and 3.0 cm outlet, the flow-rate was not as drastically effected as with the PAN membranes. The 9.0-cm probe leaked significantly, but relative recoveries were constant for each membrane length at different outlet lengths.

Experiments were carried out to compare the outlet flow with Ringer's solution and with Ringer's plus a concentrated solution of protein. The objective was to determine whether osmotic pressure would significantly influence the outlet flow independent of back-pressure from the outlet tubing. This situation has a possibility of occurring during microdialysis in blood or other tissue of high protein content. Ringer's solution was perfused through a 4.3-cm RC probe (MWCO 9000) with 50 cm of 100 μm I.D. fused-silica outlet tubing. The probe was placed in a vial containing 5 ml of constantly stirred Ringer's solution. The probe was perfused with the same solution at 5, 2.5, 1 and 0.25 μl/min and the outlet flow was calculated. The same probe was placed in a solution of 0.2 g/ml α-lactoglobulin dissolved in Ringer's solution and perfused with unmodified Ringer's solution. Osmotic pressure theory predicts the water in the probe will diffuse out into a solution of protein. This should not be dependent on perfusate flow-rate, whereas hydrodynamic pressure will be dependent on flow-rate. It was observed that when Ringer's solution was being sampled (no protein), the outlet flow decreased as the perfusion rate increased. As before this can be explained by hydrodynamic pressure. When the protein solution was being sampled, the outlet flow decreased as the perfusion rate increased too, but the loss was consistently greater at each flow-rate chosen. Osmotic leakage is dependent on membrane length: the longer the membrane the more leakage will occur. The osmotic pressure leakage is a real effect, however, for this example it is very small. The protein content of the extra cellular fluid (ECF) is relatively small (*e.g.* compared to blood) and thus the impact on actual *in vivo* sampling should not be significant.

From these results it is apparent that the choice and interrelationship of outlet tubing diameter, length of membrane, and membrane characteristics can have a significant effect on the results obtained during microdialysis sampling. Increased membrane length presents a significant advantage, in terms of relative recoveries (extraction fraction). We have found that using membranes in the range 2–5 cm recoveries plateau over a range of low flow-rates (< 2 μl/min). This is not a new observation [1,2,15,16], but has rarely been utilized in practice due to the spatial limitations of brain tissue experiments. Fig. 4 demonstrates examples of membrane length and flow-rate dependence of *in vitro* recoveries for PAN membranes using 5 μg/ml acetaminophen.

The difference between short and long membranes is dependent upon the time a plug of perfusate takes to pass through the probe. Fig. 5 illustrates how recovery varies with different lengths of membranes. In the limit of very short probes the analyte molecules diffuse across the membrane into the perfusate at a constant high rate, but are swept out of the probe before they can build up to any significant concentration. This leads to lower relative recoveries, but the highest flux across the membrane. In very long fibers the analyte concentration builds up to the point of eliminating the concentration gradient across the membrane. This leads to very high relative recoveries, with a decreasing flux along the

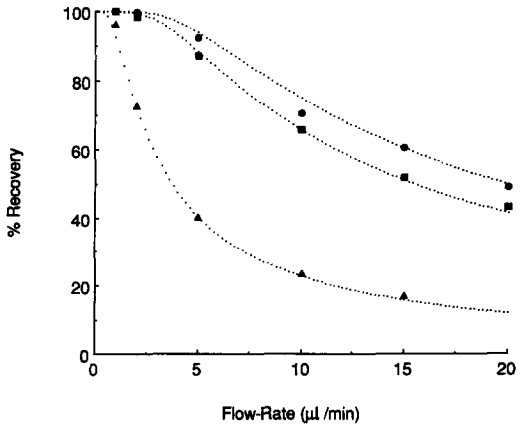

Fig. 4. *In vitro* relative recovery curves for different PAN membrane microdialysis probes (▲, 1.3 cm; ■, 5.8 cm; ● 7.5 cm).

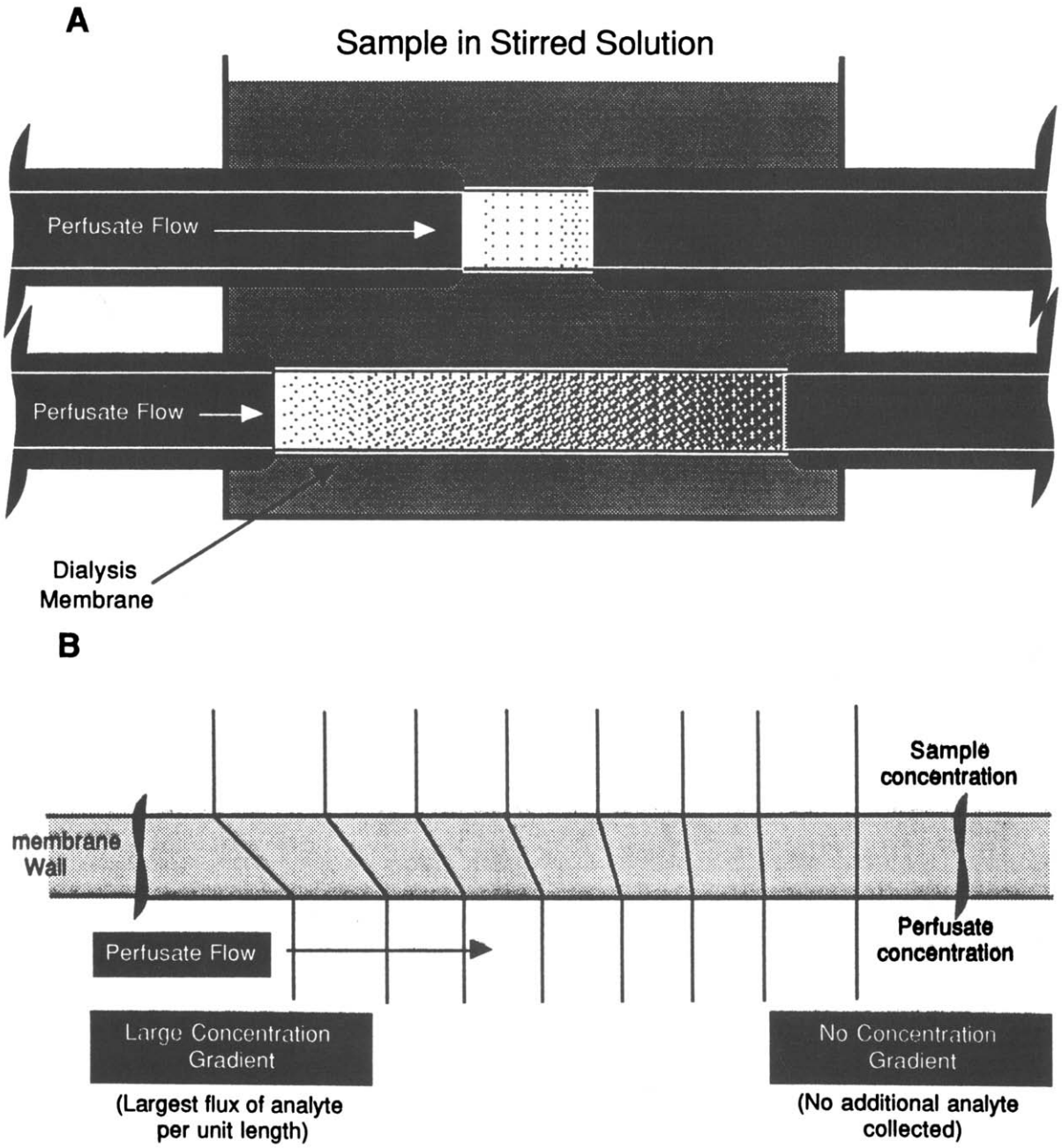

Fig. 5. (A) Comparison of short and long probes. (B) Illustration of the effects of longer mebranes and the loss of a concentration gradient in the probe.

membrane length. Although the recovery curve levels off at relatively high flow-rates *in vitro*, the same will not necessarily happen *in vivo*. Several groups [17,19] have shown that while *in vitro* the recoveries of different membranes (PAN or RC) are quite different, in tissue there is no statistical difference. While *in vitro* characteristics of a membrane can help predict its effectiveness *in vivo*, there are significant environmental differences between *in vivo* and *in vitro* microdialysis.

In tissue, the rate-limiting step for microdialysis sampling with currently available membranes is transport of analyte through the tissue [16,19]. In free solution the rate-limiting step is diffusion through the membrane. In a stirred solution, the recovery is not very dependent on the stirring rate. The same situation appears to be the case in the blood stream, where hydrodynamics (convective transport) dominates both inside and outside the membrane. With probes implanted in blood vessels it is quite possible to achieve concentration recoveries which match those *in vitro*, thus permitting the determination of the concentration of bioavailable, unbound substances [12].

Except for *in vivo* experiments conducted in blood vessels it is quite inappropriate to utilize any *in vitro* recovery data to "correct" dialysate concentrations back to tissue ECF. There have been many attempts to justify *in vitro* calibration for the determination of interstitial ECF concentrations [1,2]. In a microdialysis experiment there is not a single tissue ECF concentration, but rather a gradient of concentrations extending from the probe surface out into the tissue. The depth of this concentration gradient is very dependent on the rate of resupply and rate of metabolism of each individual substance in the ECF as well as its diffusion coefficient and the tortuosity of the tissue. A good example is the difference between *in vitro* and *in vivo* (subcutaneous tissue) recovery of acetaminophen. Using a 4-cm PAN probe at a flow-rate of 3 μl/min and 37°C the *in vitro* concentration recovery was 100%. The subcutaneous tissue recovery was only 50% when compared to ultrafiltration. Even for a longer probe at a low flow-rate, the recovery can still be limited by transport through the tissue.

In vivo recovery is sometimes estimated by the zero flux method of Smith *et al.* [11] whereby the ECF concentration is titrated with analyte to establish a point where the incoming and outgoing concentration does not change (Fig. 6). While this may work in tissue where substance resupply is fast and metabolism is slow, it is not without ambiguities. The linearity shown in Fig. 6 is likely only to be encountered at very small concentration changes. The physiology may react to counter the increased ECF concentration from the probe and thus effect the slope of the curve in Fig. 6. It also takes too long to carry out this procedure, requiring no change in analyte ECF concentration during the calibration process.

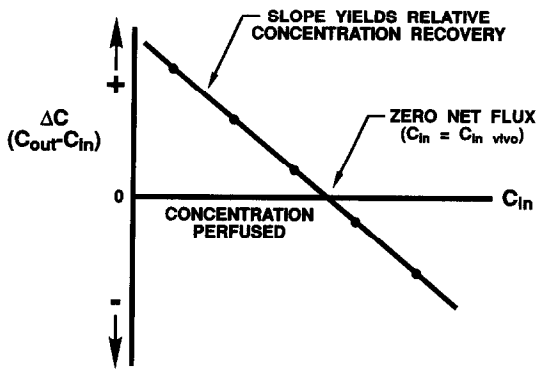

Fig. 6. Method of Smith *et al.* [11] of *in vivo* calibration.

Even if the ECF concentration could be well established, it does not tell us very much relative to the traditional *post-mortem* "concentration" in wet tissue (typically μg/g) which, of course, includes intracellular compartments broken by the homogenization process. The *in vivo* system is so heterogeneous in structure that such "concentrations" tell us very little and should not be confused with concentrations in homogeneous solutions. In general, it is the dynamics of analytes *in vivo* which is of greater interest in pharmacology than any particular concentration.

In tissue, if we can lower the transport rate (flux) across the membrane so it is not the rate-controlling step, then the tissue concentration will not be depleted near the probe as readily and concentration estimations in the ECF would perhaps become more reliable. This can be done with either more resistant membranes and/or at very low flow-rates where the inside concentration is allowed to build up, lowering the flux across the membrane at steady state. Parsons *et al.* [19] have given several examples of using very low flow-rates (0.2 μl/min) *in vivo* and extrapolating to the limit of zero flow.

Resupply of analyte to the ECF via synthesis, diffusion from the microvasculature, or release

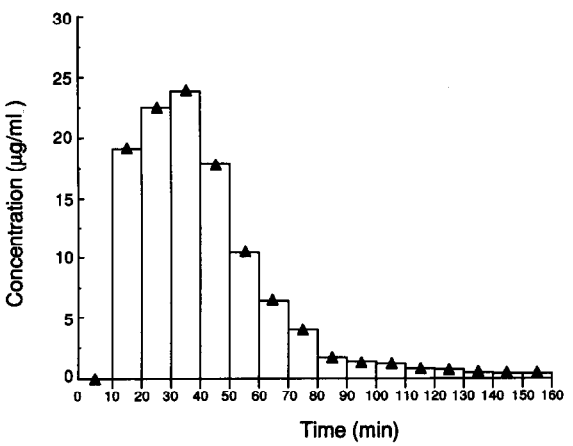

Fig. 7. Monitoring of acetaminophen in subcutaneous tissue of an awake rat. See text for experimental details.

from intracellular compartments all will have an impact on the situation for an individual analyte. Factors such as anesthesia and ambient temperature clearly influence blood flow and thus will alter the rate at which the analyte is collected from a dialysis fiber implanted in tissue. How the physiology reacts to the removal of a substance is obviously quite critical. The analyte concentration in the collected dialysate may, in fact, be more a measure of the rate of resupply than a measure of some equilibrium concentration in the ECF.

Monitoring acetaminophen, aspirin, or theophylline in blood vessels using microdialysis demonstrated its application to examining pharmacokinetics and metabolism [3]. This method determines the free concentration in the blood stream. The monitoring of drugs in the subcutaneous tissue can provide additional information about the transport of the drug of interest from the blood into the surrounding tissue. One example is illustrated in Fig. 7. The concentrations reported are perfusate concentrations and are not corrected. These results are in good agreement with previous results in blood vessels [3] and illustrate the utility of longer membranes for *in vivo* use.

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